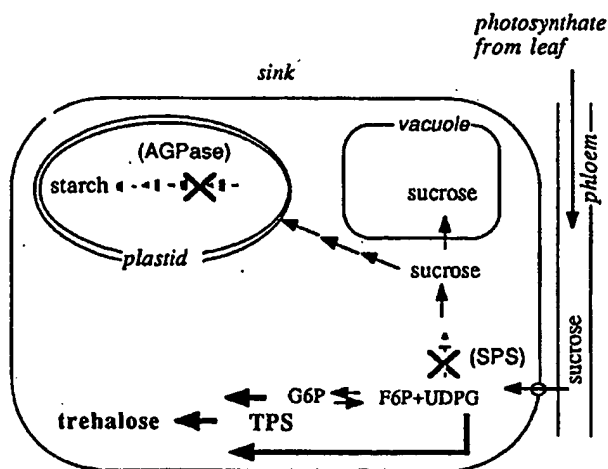




## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification <sup>6</sup> : C12N 15/82, 15/54, 15/11, 1/21, A01H 5/00, C12N 5/10, C12P 19/12, A23L 3/3562, A01N 3/00</p>	A1	<p>(11) International Publication Number: WO 95/06126</p> <p>(43) International Publication Date: 2 March 1995 (02.03.95)</p>
<p>(21) International Application Number: PCT/EP93/02290</p> <p>(22) International Filing Date: 24 August 1993 (24.08.93)</p> <p>(71) Applicant (for all designated States except US): MOGEN INTERNATIONAL N.V. [NL/NL]; Einsteinweg 97, NL-2333 CB Leiden (NL).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (for US only): HOEKEMA, Andreas [NL/NL]; Warmonderweg 66, NL-2341 KX Oegstgeest (NL). PEN, Jan [NL/NL]; Voszegge 9, NL-2318 ZJ Leiden (NL). DOES, Mirjam, Petronella [NL/NL]; Orteliusstraat 262 hs, NL-1056 PL Amsterdam (NL). VAN DEN ELZEN, Peter, J., M. [NL/NL]; Cayennehof 26, NL-2215 BH Voorhout (NL).</p> <p>(74) Agents: HUYGENS, Arthur, Victor et al.; Gist-Brocades N.V., Patents &amp; Trademarks Department, Wateringseweg 1, P.O. Box 1, NL-2600 MA Delft (NL).</p>	<p>(81) Designated States: AU, BG, BR, BY, CA, CZ, FI, HU, JP, KR, NO, NZ, PL, RO, RU, SK, UA, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</p> <p>Published With international search report.</p>	

(54) Title: PRODUCTION OF TREHALOSE IN PLANTS

ENGINEERING OF  
TREHALOSE-PRODUCTION  
IN PLANTS

## (57) Abstract

The present invention provides for the production of trehalose in a plant host due to the presence in said plant host of a plant expressible gene which comprises in sequence: (a) a transcriptional initiation region that is functional in said plant host; (b) a DNA sequence encoding a trehalose phosphate synthase activity; and optionally, (c) a transcriptional termination sequence that is functional in said plant host.

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## PRODUCTION OF TREHALOSE IN PLANTS

## FIELD OF THE INVENTION

5 This invention relates to the modification of plant carbohydrate metabolism using recombinant DNA techniques, recombinant DNA for use therein, as well as plants and parts of plants having a modified genetic constitution. Said plants may be used to extract specific carbohydrate compounds, or  
10 alternatively, they may be processed as food, feed, or ingredients thereof, having improved properties due to the presence of said carbohydrate compounds, e.g. during processing.

## STATE OF THE ART

15 Trehalose is a general name given to D-glucosyl D-glucosides which comprise disaccharides based on two  $\alpha$ -,  $\alpha$ , $\beta$ - and  $\beta$ , $\beta$ -linked glucose molecules. Trehalose, and especially  $\alpha$ -trehalose 1-(O- $\alpha$ -D-glucopyranosyl)-1'-O- $\alpha$ -D-glucopyranose) is a widespread naturally occurring  
20 disaccharide.

The chemical synthesis of trehalose is difficult (protecting groups required) and inefficient. Current natural sources of trehalose are mushrooms and the yeast *Saccharomyces cerevisiae*, that can accumulate over 10% of dry  
25 weight as trehalose. However production is hampered by high trehalase activity causing rapid metabolization of trehalose.

Elbein A.D. (1974, Adv. Carbohydrate Chem. and Biochem. 30, 227-256) gives a review of the occurrence and metabolism of the disaccharide trehalose, particularly  $\alpha$ , $\alpha$ -trehalose, in  
30 living organisms. In plants, the presence of trehalose has been reported in some lower plant species, as well as in a number of higher plant species belonging to the spermatophyta; Echinops persicus, Carex brunescens; Fagus silvaticus. However, these results have never been firmly  
35 established by other authors (e.g. Kendall et al., 1990, Phytochemistry 29, No. 8, 2525-2528). For instance, Kendall et al, supra, referring to the occurrence of trehalose in spermatophytes, stated that the presence thereof has only been firmly documented for caraway seed (Carum carvi). A  
40 report of the presence of trehalose in sunflower by Cegla et al., (1977, J. Am. Oil Chem. Soc. 54, 150 et seq.) was questioned by Kandler et al., (in: The Biochemistry of Plants

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Vol. 3 Carbohydrates: Structure and Function; Preiss, J., ed., p.228. Academic Press) according to Kendall et al, 1990, supra. Reports of trehalose in beech (Fagus sylvaticus) and cabbage could not be verified by other authors (Kendall et al., 1990, supra, and references therein).

In spite of the apparent rarity of trehalose in higher plants, the presence of trehalose degrading activities was reported for a significant number of the investigated plant families. Stable high trehalase activity was found in three wheat lines, jack pine, and Selaginella lepidophylla. Stable, low trehalase activity was found in alfalfa, black Mexican sweet corn and white spruce. Labile, moderate activities were found in two different suspensions of canola, but these could probably not be ascribed to specific trehalase activity.

Barley, brome grass, soybean and black spruce were reported to contain no trehalase activity at all (Kendall, 1990, supra).

In organisms capable of its production trehalose is believed to be biosynthesized as the 6-phosphate, whereas the storage form is the free sugar. It is therefore believed, that organisms that produce and/or store trehalose contain a phosphatase capable of cleaving trehalose 6-phosphate. (Elbein, 1974, supra). Little is known about the presence of specific trehalose phosphate phosphatases in higher plants.

#### SUMMARY OF THE INVENTION

The present invention provides for a method for the production of trehalose in a plant host due to the presence in said plant host of a plant expressible gene which comprises in sequence:

- (a) a transcriptional initiation region that is functional in said plant host,
- (b) a DNA sequence encoding a trehalose phosphate synthase activity, and optionally
- (c) a transcriptional termination sequence that is functional in said plant host.

Another embodiment of the invention comprises the production of trehalose in a plant host due to the presence in said plant host of a plant expressible gene which

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comprises in sequence:

- (a) a transcriptional initiation region that is functional in said plant host,
  - (b) a DNA sequence encoding a trehalose phosphate synthase activity, and optionally
  - (c) a transcriptional termination sequence that is functional in said plant host, and
- a plant expressible gene comprising in sequence:
- (a) a transcriptional initiation region that is functional in said plant host,
  - (b) a DNA sequence encoding an RNA sequence which is at least partially complementary to an RNA sequence which encodes a sucrose phosphate synthase enzyme (SPS) naturally occurring in said plant host, and optionally
  - (c) a transcriptional termination sequence that is functional in said plant host.

- Yet another embodiment of the invention comprises the production of trehalose in a plant host due to the presence in said plant host of a plant expressible gene which
- comprises in sequence:
- (a) a transcriptional initiation region that is functional in said plant host,
  - (b) a DNA sequence encoding a trehalose phosphate synthase activity, and optionally
  - (c) a transcriptional termination sequence that is functional in said plant host, and
- a plant expressible gene comprising in sequence:
- (a) a transcriptional initiation region that is functional in said plant host,
  - (b) a DNA sequence encoding an RNA sequence which is at least partially complementary to an RNA sequence which encodes an ADP-glucose pyrophosphorylase enzyme naturally occurring in said plant host, and optionally
  - (c) a transcriptional termination sequence that is functional in said plant host.

Yet another embodiment of the invention comprises the production of trehalose in a plant host due to the presence in said plant host of a plant expressible gene which comprises in sequence:

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- (a) a transcriptional initiation region that is functional in said plant host,
- (b) a DNA sequence encoding a trehalose phosphate synthase activity, and optionally
- 5 (c) a transcriptional termination sequence that is functional in said plant host,
- and a plant expressible gene comprising in sequence:
- (a) a transcriptional initiation region that is functional in said plant host,
- 10 (b) a DNA sequence encoding an RNA sequence at least partially complementary to an RNA sequence which encodes a sucrose phosphate synthase enzyme naturally occurring in said plant host, and optionally
- (c) a transcriptional termination sequence that is functional
- 15 in said plant host,
- and a plant expressible gene comprising in sequence:
- (a) a transcriptional initiation region that is functional in said plant host,
- (b) a DNA sequence encoding an RNA sequence at least
- 20 partially complementary to an RNA sequence which encodes an ADP-glucose pyrophosphorylase enzyme naturally occurring in said plant host, and optionally
- (c) a transcriptional termination sequence that is functional in said plant host.
- 25 The invention also extends to the plant expressible genes used in the process for making trehalose, as well as to the combinations of plant expressible genes, as well as to cloning plasmids, transformation vectors, microorganisms, an individual plant cells harboring plant expressible genes
- 30 according to the invention.
- The invention also provides a recombinant plant DNA genome which contains a plant expressible trehalose phosphate synthase gene that is not naturally present therein. The invention also comprises a recombinant plant DNA genome which
- 35 comprises a plant expressible trehalose phosphate gene that is not naturally present therein and in addition a plant expressible gene capable of inhibiting biosynthesis of an SPS activity, and/or a plant expressible gene capable of inhibiting biosynthesis of an AGPase activity.

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The invention also provides a method for obtaining a plant capable of producing trehalose comprising the steps of,

(1) introducing into a recipient plant cell a plant expressible gene comprising in sequence:

- 5 (a) a transcriptional initiation region that is functional in said plant host,
- (b) a DNA sequence encoding a trehalose phosphate synthase activity,
- (c) a transcriptional termination sequence that is
- 10 functional in said plant host, and a plant expressible gene comprising in sequence:
  - (a) a transcriptional initiation region that is functional in said plant host,
  - (b) a DNA sequence encoding a selectable marker gene that
  - 15 is functional in said plant host, and optionally
  - (c) a transcriptional termination sequence that is functional in said plant host,
- (2) generating a plant from a transformed cell under conditions that allow for selection for the presence of the
- 20 selectable marker gene.

The invention also comprises plants which produce (increased levels of) trehalose as a result of genetic modification.

The invention further comprises plants having a

25 recombinant DNA genome containing a plant expressible gene according to the invention.

The invention also comprises plants having a recombinant DNA genome containing a plant expressible gene according to the invention and which plants produce trehalose.

30 The invention also comprises plants having a recombinant DNA genome according to the invention and which exhibit increased drought resistance.

The invention also extends to parts of plants according to the invention such as cells or protoplasts or cultures

35 thereof, flowers, fruits, leaves, pollen, roots (including hairy root cultures), seeds, stalks, tubers (including so-called microtubers) and the like.

The invention also extends to a method of preserving plants or plant parts in the presence of trehalose comprising

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the steps of:

- (1) growing a plant according to the invention which produces trehalose,
- (2) harvesting the plant or plant parts which contain
- 5 trehalose, and
- (3) air drying the plants or plant parts or alternatively,
- (4) freeze drying the plants or plant parts.

The invention further comprises the plants and plant parts which have been preserved by a method according to the

10 invention.

The invention also includes a method for the production of trehalose comprising the steps of:

- (1) growing a plant which by virtue of a recombinant plant DNA genome is capable of producing (increased levels of)
- 15 trehalose,
- (2) harvesting said plant or plant part,
- (3) isolating the trehalose from the said plant or the said plant part.

The invention further includes a method for the production

20 of trehalose comprising the steps of:

- (1) growing in culture plant cells which by virtue of a recombinant plant DNA genome are capable of producing (increased levels of) trehalose,
- (2) isolating the trehalose from the said plant cell culture.

25 The invention further provides an isolated nucleic acid sequence encoding a trehalose phosphate synthase activity. A preferred isolated nucleic acid sequence is one obtained from E. coli, still more preferred is the isolated nucleic acid sequence represented in SEQIDNO: 2. Another preferred

30 embodiment comprises a nucleic acid sequence that codes for an amino acid sequence as in SEQIDNO: 3.

The following figures further illustrate the invention.

#### DESCRIPTION OF THE FIGURES.

35 Figure 1. Schematic representation of parts of the sucrose and starch biosynthetic pathways in plant sink tissues. The figure shows that carbohydrate produced in the leaf by photosynthesis is transported via the phloem tissue in the form of sucrose. Upon entering the sink it is unloaded by a



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membrane bound invertase activity to yield the monosugars glucose and fructose. By the action of a number of enzymatic steps these monosugars are converted to starch and/or sucrose as roughly shown here. The glucose metabolites G6P and UDPG are believed to be used as the substrates for the TPS-enzyme engineered into the plant by introduction of the plant expressible otsA gene. The figure shows how the amount of UDPG and G6P available as substrate is increased by reducing the levels of the enzymes SPS and AGPase. Their inhibition is marked with a cross.

Figure 2. Schematic map of the EBL4clone 7F11 from Kohara et al. (1987), containing the otsBA operon from E. coli. The 18.8 kb insert has been shaded.

15 The restriction sites for the enzymes EcoRV and HindIII used to clone the otsA gene are indicated, as well as their distance in kb with respect to the left-hand site of the insert.

20 The otsA and B gene are indicated, the arrows shows the direction of transcription. (See Fig 11, extended map).

Figure 3. Schematic representation of binary vector pMOG663.

Figure 4. Sequence of the cloned potato SPS cDNA.

25 Underscore: maize SPS cDNA sequences used as oligonucleotides in the PCR amplification reaction.

Figure 5. Schematic representation of binary vector pMOG664.

30 Figure 6. Schematic representation of binary vector pMOG665:

Figure 7. Schematic representation of binary vector pMOG666.

Figure 8. Restriction map of part of pTiB6 showing two fragments cloned in pMOG579.

35

Figure 9. Schematic representation of pMOG579 used for constructing the helper plasmid without T-region in Agrobacterium strain MOG101.

Figure 10. Schematic representation of expression vector

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pMOG180.

Figure 11. Nucleic acid sequence of the otsA gene and amino acid sequence of E. coli TPS.

5 Figure 12. Extended map of the EBL4clone 7F11 from Kohara et al. (1987), containing the otsBA operon from E. coli. The location of the TPS open reading frame (ORF) is indicated. (\*: HindIII sites not present in the map of Kohara et al., infra)

10 Figure 13. Schematic representation of binary vector pMOG799.

Figure 14. Schematic representation of binary vector pMOG801.

Figure 15. Schematic representation of binary vector pMOG802.

15

#### DETAILED DESCRIPTION OF THE INVENTION

A preferred embodiment of the invention comprises a potato plant capable of producing trehalose in tubers due to the presence in said potato plant of a plant expressible gene which comprises in sequence:

20 (a) a transcriptional initiation region derived from the 35S RNA of CaMV flanked upstream by a double enhancer,  
(b) a DNA sequence encoding trehalose phosphate synthase which is the coding region of the otsA gene located in the  
25 otsBA operon of E. coli,

(c) a transcriptional termination sequence derived from the nopaline synthase (nos) gene of Agrobacterium. Tubers of transgenic plants containing the plant expressible TPS gene produced trehalose, whereas control plants lacking this gene  
30 did not. Apparently, the trehalose phosphate which is produced by the transgenic tubers is converted into trehalose. Apparently, it is not required to provide for a trehalose phosphate phosphatase activity since it seems present in potato.

35 Also illustrated in figure 1 is an approach to improve substrate availability for TPS. To this end two genes influencing the availability of glucose-6 phosphate (G6P) and UDPG, to wit an antisense SPS gene and a antisense APGase have been cloned under the control of the CaMV 35S promoter

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for expression in plant hosts. If introduced into a plant host containing a plant expressible TPS gene according to the invention, this will increase substrate availability for TPS and therefore trehalose synthesis. It will readily occur to  
5 someone skilled in the art that also other antisense genes may be used to block the synthesis of sucrose or starch, in order to improve substrate availability.

Although the invention is described in detail for potato plants which express a plant expressible trehalose phosphate  
10 synthase gene from E. coli under the control of the CaMV 35S promoter as transcription initiation region, it will be clear to those of skill in the art that other spermatophytic plant hosts are equally suitable for the production of trehalose. Preferred plant hosts among the spermatophyta are the  
15 Angiospermae, notably the Dicotyledoneae, comprising inter alia the Solanaceae as a representative family, and the Monocotyledoneae, comprising inter alia the Gramineae as a representative family. Suitable host plants, as defined in the context of the present invention include plants (as well  
20 as parts and cells of said plants) and their progeny which have been genetically modified using recombinant DNA techniques to cause or enhance production of trehalose interest in the desired plant or plant organ; these plants may be used directly (e.g. the plant species which produce  
25 edible parts) or after the trehalose is purified from said host (which be from edible as well as inedible plant hosts). Crops with edible parts according to the invention include those which have flowers such as cauliflower (Brassica oleracea), artichoke (Cynara scolymus), fruits such as apple  
30 (Malus, e.g. domestica), banana (Musa, e.g. acuminata), berries (such as the currant, Ribes, e.g. rubrum), cherries (such as the sweet cherry, Prunus, e.g. avium), cucumber (Cucumis, e.g. sativus), grape (Vitis, e.g. vinifera), lemon (Citrus limon), melon (Cucumis melo), nuts (such as the  
35 walnut, Juglans, e.g. regia; peanut, Arachis hypogaeae), orange (Citrus, e.g. maxima), peach (Prunus, e.g. persica), pear (Pyra, e.g. communis), pepper (Solanum, e.g. capsicum), plum (Prunus, e.g. domestica), strawberry (Fragaria, e.g. moschata), tomato (Lycopersicon, e.g. esculentum), leafs,

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such as alfalfa (Medicago, e.g. sativa), cabbages (such as Brassica oleracea), endive (Cichoreum, e.g. endivia), leek (Allium, e.g. porrum), lettuce (Lactuca, e.g. sativa), spinach (Spinacia e.g. oleraceae), tobacco (Nicotiana, e.g. tabacum), roots, such as arrowroot (Maranta, e.g. arundinacea), beet (Beta, e.g. vulgaris), carrot (Daucus, e.g. carota), cassava (Manihot, e.g. esculenta), turnip (Brassica, e.g. rapa), radish (Raphanus, e.g. sativus), yam (Dioscorea, e.g. esculenta), sweet potato (Ipomoea batatas) and seeds, such as bean (Phaseolus, e.g. vulgaris), pea (Pisum, e.g. sativum), soybean (Glycin, e.g. max), wheat (Triticum, e.g. aestivum), barley (Hordeum, e.g. vulgare), corn (Zea, e.g. mays), rice (Oryza, e.g. sativa), tubers, such as kohlrabi (Brassica, e.g. oleraceae), potato (Solanum, e.g. tuberosum), and the like. The edible parts may be conserved by drying in the presence of enhanced trehalose levels produced therein due to the presence of a plant expressible trehalose phosphate synthase construct. It may be advantageous to produce enhanced levels of trehalose, by putting the DNA encoding the TPS activity under the control of an plant organ or tissue-specific promoter; the choice of which can readily be determined by those of skill in the art.

Any trehalose phosphate gene under the control of regulatory elements necessary for expression of DNA in plant cells, either specifically or constitutively, may be used, as long as it is capable of producing an active trehalose phosphate synthase activity. The nucleic acid sequence represented in SEQIDNO: 2, in fact any open reading frame encoding a trehalose phosphate synthase activity according to the invention, may be altered without necessarily altering the amino acid sequence of the protein encoded thereby. This fact is caused by the degeneracy of the genetic code. Thus the open reading frame encoding the trehalose phosphate synthase activity may be adapted to codon usage in the host plant of choice.

Also the isolated nucleic acid sequence represented by SEQIDNO: 2, may be used to identify trehalose phosphate synthase activities in other organisms and subsequently isolating them, by hybridising DNA from other sources with a

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DNA- or RNA fragment obtainable from the E. coli gene.

Preferably, such DNA sequences are screened by hybridising under stringent conditions (such as temperature and ionic strength of the hybridisation mixture. Whether or not

5 conditions are stringent also depends on the nature of the hybridisation, i.e. DNA:DNA, DNA:RNA, RNA:RNA, as well as the length of the shortest hybridising fragment. Those of skill in the art are readily capable of establishing a stringent hybridisation regime.

10 Sources for isolating trehalose phosphate synthase activities include microorganisms (e.g. bacteria, yeast, fungi), plants, animals, and the like. Isolated DNA sequences encoding trehalose phosphate activity from other sources may be used likewise in a method for producing trehalose  
15 according to the invention.

The invention also encompasses nucleic acid sequences which have been obtained by modifying the nucleic acid sequence represented in SEQIDNO: 2 by mutating one or more codons so that it results in amino acid changes in the  
20 encoded protein, as long as mutation of the amino acid sequence does not entirely abolish trehalose phosphate synthase activity.

In principle any plant host is suitable in combination with any plant expressible trehalose phosphate synthase gene.  
25 As trehalose genes from other sources become available these can be used in a similar way to obtain a plant expressible trehalose phosphate synthase gene combination as described here.

The inhibition of endogenous genes in order to enhance  
30 substrate availability for the trehalose phosphate synthase, as exemplified herein with the inhibition of endogenous sucrose phosphate synthase gene and the ADP-Glucose pyrophosphorylase gene, may be conducted in a number of ways the choice of which is not critical to the invention.  
35 Preferably gene inhibition is achieved through the so-called 'antisense approach'. Herein a DNA sequence is expressed which produces an RNA that is at least partially complementary to the RNA which encodes the enzymatic activity that is to be blocked (e.g. AGP-ase or SPS, in the examples).

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It is preferred to use homologous antisense genes as these are more efficient than heterologous genes. The isolation of an antisense SPS gene from potato using a maize SPS-gene sequence as probe serves to illustrate the feasibility of this strategy. It is not meant to indicate that, for practicing the invention the use of homologous antisense fragments is required. An alternative method to block the synthesis of undesired enzymatic activities is the introduction into the genome of the plant host of an additional copy of an endogenous gene present in the plant host. It is often observed that such an additional copy of a gene silences the endogenous gene: this effect is referred to in the literature as the co-suppressive effect, or co-suppression.

In principle both dicotyledonous and monocotyledonous plants that are amenable for transformation, can be modified by introducing a plant expressible gene according to the invention into a recipient cell and growing a new plant that harbors and expresses the plant expressible gene. Preferred plants according to the invention are those that are capable of converting trehalose-phosphate into trehalose, and which do contain no or little trehalose degrading activity. It will be understood that plants that lack the ability to convert the trehalose phosphate into trehalose are also included in the present invention. These plants may be further modified by introducing additional genes that encode phosphatases that are capable of the conversion of trehalose phosphate into trehalose. In principle also plants are envisaged that do contain trehalases, since these plants can be made suitable for the production of trehalose by inhibiting the activity of such enzymes, for instance by inhibiting expression of the genes encoding such enzymes using the antisense approach.

The method of introducing the plant expressible trehalose-phosphate gene into a recipient plant cell is not crucial, as long as the gene is stably incorporated into the genome of said plant cell. In addition to the use of strains of the genus Agrobacterium various other techniques are available for the introduction of DNA into plant cells, such as transformation of protoplasts using the calcium/polyethylene

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glycol method, electroporation and microinjection or (coated) particle bombardment (Potrykus, 1990, Bio/Technol. 8, 535-542).

In addition to these so-called direct DNA transformation methods, transformation systems involving vectors are widely available, such as viral vectors (e.g. from the Cauliflower Mosaic Virus (CaMV) and bacterial vectors (e.g. from the genus Agrobacterium) (Potrykus, 1990, Bio/Technol. 8, 535-542). After selection and/or screening, the protoplasts, cells or plant parts that have been transformed can be regenerated into whole plants, using methods known in the art (Horsch et al., 1985, Science 225, 1229-1231).

It has been shown that monocotyledonous plants are amenable to transformation and that fertile transgenic plants can be regenerated from transformed cells. The development of reproducible tissue culture systems for these crops, together with the powerful methods for introduction of genetic material into plant cells has facilitated transformation. Presently, preferred methods for transformation of monocots are microprojectile bombardment of explants or suspension cells, and direct DNA uptake or electroporation (Shimamoto, et al., 1989, Nature 338, 274-276). Transgenic maize plants have been obtained by introducing the Streptomyces hygrosopicus bar-gene, which encodes phosphinothricin acetyltransferase (an enzyme which inactivates the herbicide phosphinothricin), into embryogenic cells of a maize suspension culture by microprojectile bombardment (Gordon-Kamm, 1990, Plant Cell, 2, 603-618). The introduction of genetic material into aleurone protoplasts of other monocot crops such as wheat and barley has been reported (Lee, 1989, Plant Mol. Biol. 13, 21-30). Wheat plants have been regenerated from embryogenic suspension culture by selecting only the aged compact and nodular embryogenic callus tissues for the establishment of the embryogenic suspension cultures (Vasil, 1990 Bio/Technol. 8, 429-434). The combination with transformation systems for these crops enables the application of the present invention to monocots. These methods may also be applied for the transformation and regeneration of dicots.

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Monocotyledonous plants, including commercially important crops such as corn are amenable to DNA transfer by Agrobacterium strains (European patent 159 418 B1; Gould J, Michael D, Hasegawa O, Ulian EC, Peterson G, Smith RH, (1991) 5 Plant. Physiol. 95, 426-434).

As regards the choice of the host plant it is preferred to select plant species with little or no trehalose degrading activity. However, plants that do exhibit trehalase activity are not excluded from being a suitable host plant for the 10 production of trehalose, although it may be necessary to provide for inhibition of trehalase activity if this prevents the accumulation of trehalose altogether. Such inhibition can be achieved using the antisense approach well known in the art, and illustrated for other purposes in this 15 specification.

It should also be understood that the invention is not limited to the use of the CaMV 35S promoter as transcription initiation region. Suitable DNA sequences for control of expression of the plant expressible genes, including marker 20 genes, such as transcriptional initiation regions, enhancers, non-transcribed leaders and the like, may be derived from any gene that is expressed in a plant cell which, such as endogenous plant genes, genes naturally expressed in plant cells such as those located on wild-type T-DNA of 25 Agrobacterium, genes of plant viruses, as well as other eukaryotic genes that include a transcription initiation region that conforms to the consensus sequence for eukaryotic transcription initiation. Also intended are hybrid promoters combining functional portions of various promoters, or 30 synthetic equivalents thereof. Apart from constitutive promoters, inducible promoters, or promoters otherwise regulated in their expression pattern, e.g. developmentally or cell-type specific, may be used to control expression of the plant expressible genes according to the invention as 35 long as they are expressed in plant parts that contain substrate for TPS.

To select or screen for transformed cells, it is preferred to include a marker gene linked to the plant expressible gene according to the invention to be transferred to a plant cell.



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The choice of a suitable marker gene in plant transformation is well within the scope of the average skilled worker; some examples of routinely used marker genes are the neomycin phosphotransferase genes conferring resistance to kanamycin (EP-B 131 623), the Glutathion-S-transferase gene from rat liver conferring resistance to glutathione derived herbicides (EP-A 256 223), glutamine synthetase conferring upon overexpression resistance to glutamine synthetase inhibitors such as phosphinothricin (WO87/05327), the acetyl transferase gene from Streptomyces viridochromogenes conferring resistance to the selective agent phosphinothricin (EP-A 275 957), the gene encoding a 5-enolshikimate-3-phosphate synthase (EPSPS) conferring tolerance to N-phosphonomethylglycine, the bar gene conferring resistance against Bialaphos (e.g. WO91/02071) and the like. The actual choice of the marker is not crucial as long as it is functional (i.e. selective) in combination with the plant cells of choice.

The marker gene and the gene of interest do not have to be linked, since co-transformation of unlinked genes (U.S. Patent 4,399,216) is also an efficient process in plant transformation.

Preferred plant material for transformation, especially for dicotyledonous crops are leaf-discs which can be readily transformed and have good regenerative capability (Horsch R.B. et al., (1985) Science 227, 1229-1231).

Whereas the production of trehalose can be achieved with the plant expressible trehalose phosphate synthase gene as the sole carbohydrate modifying gene, the invention is further illustrated with examples of additional plant expressible antisense genes that are capable of effecting an increase of the availability of the substrate for trehalose phosphate synthase. Specific examples of such genes are the plant expressible antisense genes for SPS from maize and potato and AGPase from potato. The down regulation of carbohydrate modifying enzymes using the antisense approach is not limited by the specific examples. For instance partially complementary plant expressible antisense genes can be used to inhibit expression of a target gene, as long as

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the plant expressible antisense gene produces a transcript that is sufficiently complementary with the transcript of the target gene and sufficiently long to inhibit expression said target gene.

5 It is immaterial to the invention how the presence of two or more genes in the same plant is effected. This can inter alia done be achieved by one of the following methods:

- (a) transformation of the plant line with a multigene construct containing more than one gene to be introduced,
- 10 (b) co-transforming different constructs to the same plant line simultaneously,
- (c) subsequent rounds of transformation of the same plant with the genes to be introduced,
- (d) crossing two plants each of which contains a different
- 15 gene to be introduced into the same plant.

The field of application of the invention lies both in agriculture and horticulture, for instance due to improved properties of the modified plants as such, as well as in any form of industry where trehalose is or will be applied.

20 Trehalose phosphate and trehalose can be used as such for instance in purified form or in admixtures, or in the form of a storage product in plant parts. Plant parts harboring (increased levels of) trehalose phosphate or trehalose may be used as such or processed without the need to add trehalose.

25 Also trehalose can be purified from the plants or plant parts producing it subsequently used in an industrial process. In the food industries trehalose can be employed by adding trehalose to foods before drying. Drying of foods is an important method of preservation in the industry.

30 Trehalose seems especially useful to conserve food products through conventional air-drying, and to allow for fast reconstitution upon addition of water of a high quality product (Roser et al, July 1991, Trends in Food Science and Technology, pp. 166-169). The benefits include retention of

35 natural flavors/fragrances, taste of fresh product, and nutritional value (proteins and vitamins). It has been shown that trehalose has the ability to stabilize proteins and membranes, and to form a chemically inert, stable glass. The low water activity of such thoroughly dried food products

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prevents chemical reactions, that could cause spoilage.

Field crops like corn, cassava, potato, sugar beet and sugarcane have since long been used as a natural source for bulk carbohydrate production (starches and sucrose). The  
5 production of trehalose in such crops, facilitated by genetic engineering of the trehalose-biosynthetic pathway into these plant species, would allow the exploitation of such engineered crops for trehalose production.

All references cited in this specification are indicative  
10 of the level of skill in the arts to which the invention pertains. All publications, whether patents or otherwise, referred to previously or later in this specification are herein incorporated by reference as if each of them was individually incorporated by reference.

15 The Examples given below are just given for purposes of enablement and do not intend in any way to limit the scope of the invention.

#### EXPERIMENTAL

##### 20 DNA manipulations

All DNA procedures (DNA isolation from E.coli, restriction, ligation, transformation, etc.) are performed according to standard protocols (Sambrook et al. (1989) Molecular Cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory  
25 Press, CSH, New York).

##### Strains

In all examples E.coli K-12 strain DH5 $\alpha$  is used for cloning. The Agrobacterium tumefaciens strain used for plant  
30 transformation experiments is MOG101 which is a non-oncogenic octopine type helper strain derived from LBA1010 (Koekman et al. (1982) Plasmid 7, 119) by substitution of the T-DNA by a spectinomycin resistance marker.

##### 35 Construction of Agrobacterium strain MOG101

A binary vector system (Hoekema A., Hirsch, P.R., Hooykaas, P.J.J., and Schilperoort, R.A. (1983) Nature 303, 179) is used to transfer gene constructs into potato plants. The helper plasmid conferring the Agrobacterium tumefaciens

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virulence functions is derived from the octopine Ti-plasmid pTiB6. MOG101 is an Agrobacterium tumefaciens strain carrying a non-oncogenic Ti-plasmid (Koekman et al. 1982, supra) from which the entire T-region is deleted and substituted by a  
5 bacterial Spectinomycin resistance marker from transposon Tn1831 (Hooykaas et al., 1980 Plasmid 4, 64-75).

The Ti-plasmid pTiB6 contains two adjacent T-regions, TL (T-left) and TR (T-right). To obtain a derivative lacking the TL- and TR-regions, we constructed intermediate vector  
10 pMOG579. Plasmid pMOG579 is a pBR322 derivative which contains 2 Ti-plasmid fragments homologous to the fragments located left and right outside the T-regions of pTiB6 (shaded in Figures 8 and 9). The 2 fragments are separated in pMOG579 by a 2.5 kb BamHI - HindIII fragment from transposon Tn1831  
15 (Hooykaas et al., 1980 Plasmid 4, 64-75) carrying the spectinomycin resistance marker (Figure 9). The plasmid is introduced into Agrobacterium tumefaciens strain LBA1010 [C58-C9 (pTiB6) = a cured C58 strain in which pTiB6 is introduced (Koekman et al. (1982), supra), by triparental  
20 mating from E.coli, using HB101 8pRK2013 as a helper. Transconjugants are selected for resistance to Rifampicin (20 mg/l) and spectinomycin (250 mg/l). A double recombination between pMOG579 and pTiB6 resulted in loss of carbenicillin resistance (the pBR322 marker) and deletion of the entire T-  
25 region. Of 5000 spectinomycin resistant transconjugants replica plated onto carbenicillin (100 mg/l) 2 are found sensitive. Southern analysis (not shown) showed that a double crossing over event had deleted the entire T-region. The resulting strain is called MOG101. This strain and its  
30 construction is analogous to strain GV2260 (Deblaere et al. 1985, Nucl. Acid Res. 13, 4777-4788).

An alternative helper strain for MOG101 is e.g. LBA4404; this strain can also suitably be used for introduction of a binary plasmid, such as pMOG799 and subsequent plant  
35 transformation. Other suitable helper strains are readily available.

#### Construction of the expression vector pMOG180

The expression vector pMOG180 is a derivative of pMOG18

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(EP 0 479 359 A1, Example 2b) wherein the gene coding for GUS is removed and other genes can be inserted between the ALMV RNA4 leader and 3' nos terminator as a BamHI fragment.

For this purpose, the EcoRI/NcoI fragment from pMOG18, containing the 35S promoter and ALMV RNA4 leader sequences is synthesized using PCR technology with the primer sets 5' GTTCTACAGGACGGAGGATCCTGGAAGTATTTGAAAGA 3' and 5' CAGCTATGACCATGATTACG 3' thus mutating the NcoI site into a BamHI site. pMOG18 vector is then cut with EcoRI and BamHI after which the newly synthesized EcoRI/BamHI fragment can be ligated between these restriction sites. To circumvent PCR-induced random mutations in the promoter sequences, the EcoRI/EcoRV fragment in the PCR synthesized EcoRI/BamHI fragment is replaced by wildtype sequences from pMOG18. The short EcoRV/BamHI is checked for mutations by sequencing. The resulting expression vector is plasmid pMOG180 (Figure 10):

#### Triparental matings

The binary vectors pMOG663-666 are mobilized in triparental matings with the E. coli strain HB101 containing plasmid pRK2013 (Ditta G., Stanfield, S., Corbin, D., and Helinski, D.R. et al. (1980) Proc. Natl. Acad. Sci. USA 77, 7347) into *Agrobacterium tumefaciens* strain MOG101 and used for transformation.

25

#### Transformation of potato

Potato (*Solanum tuberosum* cv. Désiree) is transformed with the *Agrobacterium tumefaciens* strain MOG101 containing the binary vector of interest as described (Hoekema A., Huisman, M.J., Molendijk, L., Van den Elzen, P.J.M., and Cornelissen, B.J.C. (1989) Bio/technology 7, 273). The basic culture medium is MS30R30, consisting of MS-medium (Murashige, T., and Skoog, F. (1962) Physiol. Plan. 14, 473), supplemented with 30 g/L sucrose, R3 vitamins (Ooms et al. G., Burrell, M.M., Karp, A., Bevan, M., and Hille, J. (1987) Theor. Appl. Genet. 73, 744), 5  $\mu$ M zeatin riboside (ZR), and 0.3  $\mu$ M indole acetic acid (IAA). The media are solidified where necessary, with 0.7 g/L Daichin agar.

Tubers of *Solanum tuberosum* cv. Désiree are peeled and

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surface sterilized for 20 minutes in 0.6% hypochlorite solution containing 0.1% Tween-20. The potatoes are washed thoroughly in large volumes of sterile water for at least 2 hours. Discs of approximately 2 mm thickness are sliced from cylinders of tuber tissue prepared with a corkbore. Discs are incubated for 20 minutes in a suspension consisting of the MS30R3 medium without ZR and IAA, containing  $10^6$ - $10^7$  bacteria/ml of Agrobacterium MOG101 containing the binary vector. The discs are subsequently blotted dry on sterile filter paper and transferred to solid MS30R3 medium with ZR and IAA. Discs are transferred to fresh medium with 100 mg/L cefotaxim and 50 mg/L vancomycin after 2 days. A week later, the discs are transferred again to the same medium, but this time with 100 mg/L kanamycin to select for transgenic shoots. After 4-8 weeks, shoots emerging from the discs are excised and placed onto rooting medium (MS30R3-medium without ZR and IAA, but with 100 mg/L cefotaxim and 100 mg/L kanamycin). The shoots are propagated axenically by meristem cuttings and transferred to soil after root development. Where appropriate, 10 mg/L hygromycin is used for selection instead of 100 mg/L kanamycin.

#### Trehalose assay

Trehalose is determined essentially as described by Hottiger et al. (Hottiger et al. (1987) J. Bact. 169, 5518). Potato tuber tissue is frozen in liquid nitrogen, powdered with pestle and mortar and subsequently extracted for 60 minutes at room temperature in app. 3 volumes of 500 mM trichloroacetic acid. After centrifugation the pellet is extracted once more in the same way. The combined supernatants from the two extractions are assayed for anthrone positive material (Spiro R.G. (1966) Meth. Enzymol. 8, 3). Trehalose is determined qualitatively by TLC. The extracts are deionized (Merck, Ion exchanger V) and loaded onto Silica Gel 60 plates (Merck). After chromatography plates are developed with n-butanol-pyridine-water (15:3:2, v/v). Spots are visualized by spraying with 5 mg/ml vanillin in concentrated  $H_2SO_4$  and heating at 130°C. Commercially available trehalose (Sigma) is used as a standard.

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Enzyme assays

In all determinations non-transgenic tuber material of variety Desiree is used as control. Protein content in all samples is determined as described by Bradford (Bradford  
5 (1976) Anal. Biochem. 72, 248). For assays on tuber extracts, frozen potato tuber slices of app. 100 mg are homogenized in 100  $\mu$ l 20 mM HEPES pH 7.4, centrifuged (Eppendorf, 5 minutes at maximum speed). The supernatant is used for activity assays.

10

TPS activity - TPS activity is determined essentially as described by Hottiger et al. (Hottiger T., Schmutz, P., and Wiemken, A. (1987) J. Bact. 169, 5518). Tuber extract assay mixtures contained 50 mM tricine ( $K^+$ ) pH 7.0, 10 mM glucose-  
15 6-phosphate, 5mM UDP-glucose, 12.5 mM  $MgCl_2$ , in a total volume of 0.4 ml. In controls glucose-6-phosphate is omitted. Assay mixtures are incubated at 37°C for 5-30 min. The reaction is stopped by addition of 0.2 ml ice-cold 1 N perchloric acid. After neutralization with 0.2 ml 1 N KOH,  
20 the samples are stored on ice for 10 minutes and subsequently centrifuged at 2,000 x g. UDP is determined in the supernatants. The assay mixture contained 140 mM tricine ( $K^+$ ) pH 7.6, 2 mM phosphoenolpyruvate, 0.31 mM NADH, 20 U lactate dehydrogenase from rabbit muscle (Sigma Type XXXIX) in a  
25 total volume of 1.96 ml. The reaction is started by addition of 20 U pyruvate kinase from rabbit muscle (Sigma Type III). The decrease of the absorbance at 340 nm at 37°C is used to calculate the UDP concentration. One unit of TPS activity is defined as nmol UDP formed per min at 37°C.

30

AGPase activity - AGPase activity is determined as described by Müller-Röber et al. (Müller-Röber B., Sonnewald, U., and Willmitzer, L. (1992) EMBO J. 11, 1229). Production of glucose-1-phosphate from ADP-glucose is determined in a NAD-  
35 linked glucose-6-phosphate dehydrogenase system. The reaction assay contained 80 mM HEPES pH 7.4, 10 mM  $MgCl_2$ , 1 mM ADP-glucose, 0.6 mM NAD, 10  $\mu$ M glucose-1,6-diphosphate, 3 mM DTT, 0.02% bovine serum albumin, 1 U phosphoglucomutase from rabbit muscle (Sigma), 2.5 U NAD-linked glucose-6-phosphate

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dehydrogenase from *Leuconostoc mesenteroides* and tuber extract. The reaction is initiated by addition of sodiumpyrophosphate to a final concentration of 2 mM. NAD reduction is measured spectrophotometrically at 340 nm and 30°C. A unit of AGPase activity is defined as nmol glucose-1-phosphate generated per min at 30°C.

SPS activity - SPS activity is determined essentially as described by Lunn & ApRees (Lunn and ApRees (1990) *Phytochem.* 29, 1057). Assay mixtures contained 50 mM tricine (K<sup>+</sup>) pH 7.0, 5 mM fructose-6-phosphate, 5mM UDP-glucose, 12.5 mM MgCl<sub>2</sub>, tuber extract, and water in a total volume of 0.4 ml. In controls fructose-6-phosphate is omitted. Assay mixtures are incubated at 25°C for 5-30 min. The reaction is stopped by addition of 0.2 ml ice-cold 1 N perchloric acid. After neutralization with 0.2 ml 1 N KOH, the samples are stored on ice for 10 minutes and subsequently centrifuged at 2,000 x g. UDP is determined in the supernatants. The assay mixture contained 140 mM tricine (K<sup>+</sup>) pH 7.6, 2 mM phosphoenolpyruvate, 0.31 mM NADH, 20 U lactate dehydrogenase from rabbit muscle (Sigma Type XXXIX) in a total volume of 1.96 ml. The reaction is started by addition of 20 U pyruvate kinase from rabbit muscle (Sigma Type III). The decrease of the absorbance at 340 nm at 37°C is used to calculate the UDP concentration. One unit of SPS activity is defined as nmole UDP formed per min at 37°C.

#### EXAMPLE I

##### Cloning of the *Escherichia coli* otsA gene

In *E.coli* trehalose phosphate synthase (TPS) is encoded by the otsA gene located in the operon otsBA. The location and the direction of transcription of this operon on the *E.coli* chromosome are precisely known (Kaassen I., Falkenberg, P., Styrvold, O.B., and Strom, A.R. (1992) *J. Bact.* 174, 889). It is located in the 41-42' region of the *E.coli* chromosome, and is confined on a 2.9 kb HindIII fragment on EMBL4 genomic clone designated 7F11 of the map by Kohara et al. (Kohara Y., Akiyama, K. and Isono, K. (1987) *Cell* 50, 495). The position of the otsBA operon on this clone 7F11 is shown in Figure 2.



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DNA is prepared from a lysate of lclone 7F11, and digested with HindIII. We isolated the 2.9 kb HindIII fragment containing otsBA (the 'righthand' HindII-site at 14.3 kb in the insert is omitted on the map by Kohara, as already noticed by Kaassen). The 2.9 kb HindIII-fragment is cloned in pUC18 linearized with HindIII. From the resulting plasmid an EcoRV/HindIII fragment of 2.1 kb containing the otsA gene is isolated, it is made blunt using Klenow polymerase and then cloned in vector pMOG180 linearized with BamHI and made blunt using Klenow polymerase. The resulting expression plasmid contained the E. coli otsA gene in the correct orientation under control of the Cauliflower Mosaic Virus (CaMV) 35S promoter with double enhancer (Guilley H., Dudley, R.K., Jonard, G., Balazs, E., and Richards, K.E. (1982) Cell 30, 763), the Alfalfa Mosaic Virus (AlMV) RNA4 leader sequence (Brederode et al. F.T., Koper-Zwarthoff, E.C., and Bol, J.F. (1980) Nucl. Acids Res. 8, 2213) and the nopaline synthase transcription terminator sequence from Agrobacterium tumefaciens. The expression cassette is cloned as an EcoRI/HindIII fragment into the binary vector pMOG23 (deposited on January 29, 1990 at the Centraal Bureau voor Schimmelcultures under accession number 102.90) The resulting binary vector pMOG663 (see Figure 3) is used to transform potato.

25

#### Example II

##### Trehalose production in potato tubers transformed with pMOG663.

Potato tuber discs are transformed with the binary vector pMOG663. Transgenic shoots are selected on kanamycin. A number of 20 independent transgenic shoots containing the plant expressible E.coli TPS-construct are analyzed for trehalose phosphate synthase (TPS) activity. Shoots found to contain the enzyme are grown to mature plants. Mature tubers of those transgenic potato plants, analyzed for trehalose, are found to contain elevated levels of trehalose in comparison with non-transgenic control plants. Transgenic plant line 663.1 is propagated for further work.

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**Example III****Construction of pMOG664**

Two oligonucleotides corresponding to the cDNA sequence of the small subunit of ADP-glucose pyrophosphorylase (AGPase) from potato tuber (EMBL data bank accession number X61186) are synthesized. The sequences are as follows:

5' TCCCATGGAATCAAAGCATCC 3' (SEQIDNO: 4)

5' GATTGGATCCAGGGCACGGCTG 3' (SEQIDNO: 5)

10 The oligonucleotides are designed to contain suitable restriction sites (BamHI and NcoI, underlined) at their termini to allow assembly in an expression cassette in an antisense orientation. A fragment of about 1 kb is PCR amplified with these oligonucleotides using DNA isolated from a cDNA library from potato cv. Désiree prepared from 2 month old leaf tissue (Clontech) as a template. After sequencing it can be shown, that the fragment is identical with the AGPase sequence deposited in the EMBL data bank. Following digestion with BamHI and NcoI, the fragment is cloned in pMOG18 linearized with BamHI and NcoI. From the resulting plasmid the 1.85 kb EcoRI/BamHI fragment is isolated (containing the CaMV 35S promoter, the AlMV RNA4 leader and the AGPase fragment in an antisense orientation) as well as the 0.25 kb BamHI/HindIII fragment containing the nos-terminator. These two fragments are cloned in a three-way ligation with the binary vector pMOG22 linearized with EcoRI and HindIII. The binary vector pMOG22 contains a plant expressible HPTII gene for hygromycin selection in transgenic plants (pMOG22 has been deposited at the Centraal Bureau voor Schimmelcultures on January 29, 1990 under accession number 101.90). The resulting binary vector pMOG664 (see Figure 4) is used for potato transformation.

**Example IV****Construction of pMOG665**

A set of oligonucleotides complementary to the sequence of the maize sucrose phosphate synthase (SPS) cDNA (Worrell A.C., Bruneau, J-M., Summerfelt, K., Boersig, M., and Voelker, T.A. (1991) Plant Cell 3, 1121) is synthesized.

- 25 -

Their sequences are as follows:

5' CTAGGTCGTGATTCTGATACAGGTGGCCAGGTG 3' (SEQIDNO: 6)

5' CAGCATCGGCATAGTGCCCATGTATCACGTAAGGC 3' (SEQIDNO: 7)

These oligonucleotides are used to PCR amplify a DNA fragment of 370 bp using DNA isolated from a potato cv. Désirée cDNA library prepared from 2 month old leaf tissue (Clontech) as a template. After sequencing of this fragment it can be shown that it is highly complementary to the SPS sequence of maize (see Figure 5, and Worrell et al. (1991) Plant Cell 3, 1121). The PCR amplified fragment is made blunt-ended and cloned in pMOG18 linearized with NcoI and BamHI and made blunt-ended with Klenow polymerase. From a clone with the SPS fragment in the antisense orientation with respect to the CaMV 35S promoter, the EcoRI/HindIII fragment is cloned into the binary vector pMOG22 linearized with EcoRI, in a three-way ligation using a synthetic adapter with the following sequence:

5' AGCTTCCCCCCCCG 3' (SEQIDNO: 16)

|||||

20 3' AGGGGGGGCTTAA 5' (SEQIDNO: 17)

The resulting binary vector pMOG665 (see Figure 6) is used for potato transformation.

25

#### Example IV

##### Construction of pMOG666

The EcoRI fragment of plasmid pMOG665 containing the antisense SPS cassette, is cloned in the binary vector pMOG664 (containing the antisense AGPase cassette) linearized with EcoRI. The resulting binary vector carrying the two anti-sense constructs is called pMOG666 (see Figure 7).

35

#### Example V

##### Trehalose production in potato transformed with pMOG663 and pMOG664

Potato tuber discs of kanamycin resistant transgenic plant line 663.1, expressing TPS (example II) are transformed with the binary vector pMOG664, containing the antisense AGPase construct. Transgenic shoots are selected on 10 mg/L

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hygromycin. Transgenic shoots are recovered, and checked by PCR for the presence of both pMOG663 and pMOG664 sequences. Transgenic plants containing the plant expressible E. coli TPS construct and the antisense AGPase construct are analyzed  
5 for TPS and AGPase activity.

Analysis of transgenic tubers for AGPase activity shows reductions in activity levels in individual transgenic lines in comparison with non-transgenic controls. Northern blotting shows that also mRNA levels for AGPase are reduced in the  
10 transgenic plants compared to those in non-transgenic control plants. Trehalose levels in tubers of transgenic potato plants, found to exhibit TPS activity, and having reduced levels of AGPase, show an increase in comparison with the levels that can be found in tubers of transgenic plant line  
15 663.1.

#### Example VI

##### Trehalose production in potato transformed with pMOG663 and 20 pMOG665

Potato tuber discs of transgenic plant line 663.1 expressing TPS are transformed with the binary vector pMOG665, containing the antisense SPS construct. Transgenic shoots are selected on 10 mg/L hygromycin. Emerging shoots are checked  
25 by PCR for the presence of both pMOG663 and pMOG665 sequences. Transgenic shoots containing the plant expressible E. coli TPS construct and the antisense SPS construct are analyzed for TPS and SPS activity.

30 Analysis of transgenic tubers for SPS activity shows reductions in the levels for both enzymes in individual transgenic lines in comparison with non-transgenic controls. Northern blotting shows that also mRNA levels for SPS are reduced in the transgenic plants compared to those in non-  
35 transgenic control plants. Trehalose levels in tubers of transgenic potato plants, found to exhibit TPS activity, and having reduced levels of SPS, show an increase in comparison with the levels found in tubers of transgenic plant line 663.1.

**Example VII****Trehalose production in potato transformed with pMOG663 and pMOG666**

- 5 Potato tuber discs of transgenic plant line 663.1 expressing TPS are transformed with the binary vector pMOG666, containing the two antisense AGPase and SPS constructs. Transgenic shoots are selected on 10 mg/L hygromycin. Emerging shoots are checked by PCR for the presence of the
- 10 plant expressible *E. coli* TPS construct, and the antisense AGPase and SPS construct. Positive shoots are analyzed for TPS, AGPase and SPS activity.

- Analysis of transgenic tubers for AGPase and SPS activity
- 15 shows reductions in the levels for both enzymes in individual transgenic lines in comparison with non-transgenic controls. Northern blotting shows that also mRNA levels are reduced in the transgenic plants compared to those in non-transgenic control plants. Trehalose levels in tubers of transgenic
- 20 potato plants, found to exhibit TPS activity, and having reduced levels of AGPase and SPS, show an increase in comparison with the levels found in tubers of transgenic plant line 663.1.

- 25 The following examples describe the identification of the nucleotide sequence encoding a full length *E.coli* trehalose phosphate synthase activity. The amino acid sequence of the complete *E. coli* TPS is also disclosed.

30

**Example VIII****Cloning of a full length *E. coli* otsA gene**

- In *E.coli* trehalose phosphate synthase (TPS) is encoded by the *otsA* gene located in the operon *otsBA*. The location and the direction of transcription of this operon on the *E.coli*
- 35 chromosome are known (Kaasen, I., Falkenberg, P., Styrvold, O.B., and Ström, A.R. (1992) J. Bact. 174, 889). The *otsA* gene is located at 42', and according to Kaasen et al. confined on a 18.8 kb fragment present in the EMBL4 genomic clone designated 7F11 of the map by Kohara et al. (Kohara,

Y., Akiyama, K., and Isono, K. (1987) Cell 50, 495). DNA prepared from a lysate of lambda clone 7F11, and digested with HindIII. The isolated 2.9 kb HindIII fragment (the 'right-hand' HindIII site at 14.3 kb in the insert was omitted on the map by Kohara et al., as already noticed by Kaasen et al.) is cloned in pUC18 linearized with HindIII. The 2.9 kb HindIII insert from the resulting plasmid, designated pMOG674, is sequenced. The sequence is found to contain part of the araH gene of the arabinose transport operon (Scripture, J.B., Voelker, C., Miller, S., O'Donnell, R.T., Polgar, L., Rade, J., Horazdovsky, B.F., and Hogg, R.W. (1987) J. Mol. Biol. 197, 37), the otsB gene encoding TPP as localized by Kaasen et al. and part of the otsA gene encoding TPS. The otsA is found not to be confined to the 2.9 kb HindIII fragment as described by Kaasen et al. To complete the sequence an overlapping BamHI/EcoRI fragment is isolated and partially sequenced. The complete TPS-encoding sequence of the otsA gene is shown in Figure 11 (SEQIDNO: 2). The position of the otsA gene on clone 7F11, with the restriction enzyme sites used, is shown in Figure 12. An additional HindIII site not present on the map published by Kohara et al. is found on the 'left-hand' site of the 2.9 kb HindIII fragment.

The HindIII site in pMOG180 is replaced by a SstI site, by cloning the oligonucleotide duplex:

SstI  
 5' AGCTCAGGAGCTCTCAGG 3' (SEQIDNO: 8)  
 3' GTGCTCGAGAGTCCTCGA 5' (SEQIDNO: 9)

into pMOG180 cut with HindIII. The resulting vector is designated pMOG746. The oligonucleotide duplex:

BamHI      SmaI      SphI      HindIII      BamHI


35 5' GATCCCCCGGGGCATGCAAGCTTG 3' (SEQIDNO: 10)  
 3' GGGGCCCCGTACGTTTGAACCTAG 5' (SEQIDNO: 11)

is cloned in vector pMOG746 linearized with BamHI. The vector with the oligonucleotide duplex in the desired orientation (checked by restriction enzyme digestion) is designated

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pMOG747. The 2.9 kb HindIII fragment of plasmid pMOG674 is cloned in pMOG747 linearized with HindIII, resulting in vector pMOG748. The app. 2.4 kb EcoRV/SstI and the app. 3.5 kb SstI/SmaI fragments of pMOG748 are isolated, ligated and transformed into *E. coli*, thus deleting the 3' end of the 2.9 kb HindIII fragment. The resulting plasmid is designated pMOG749. The 5' end of the *otsA* gene is synthesized by PCR using the synthetic oligonucleotides TPS1 and TPS2 with pMOG749 as a template.

10

TPS1 5' GAGAAAATACCCGGGGTGATGAC 3' (SEQIDNO: 12)

TPS2 5' GATAATCGTGGATCCAGATAATGTC 3' (SEQIDNO: 13)

By sequencing it is confirmed that the 0.4 kb PCR fragment has the correct sequence. The 1 kb BamHI/HindIII fragment of pMOG749 is cloned together with the 0.4 kb XmaI/BamHI PCR fragment in pMOG747 linearized with XmaI and HindIII. In the resulting plasmid, digested with HindIII and SstI, the synthetic oligonucleotide duplex TPS6/7 is cloned, encoding the three C-terminal amino acids of TPS.

20

LysLeuAlaStop

5' AGCTGGCGTGAGGAGCGGTTAATAAGCTTGAGCT 3' (SEQIDNO: 14)

3' CCGCACTCCTCGCCAATTATTCGAAC 5' (SEQIDNO: 15)

25

In the resulting plasmid, digested with HindIII and SstI, the 0.25 kb HindIII/SstI fragment of plasmid pMOG749 is cloned, comprising the terminator from the *Agrobacterium tumefaciens* nopaline synthase (NOS) gene, resulting in plasmid pMOG798. This plasmid contains the *E. coli otsA* gene in the correct orientation under control of the Cauliflower Mosaic Virus (CaMV) 35S promoter with double enhancer (Guilley et al. (1982) Cell 30, 763), the Alfalfa Mosaic Virus (AMV) RNA4 leader sequence (Brederode et al. (1980) Nucl. Acids Res. 8, 2213) and the nopaline synthase transcription terminator sequence from *Agrobacterium tumefaciens*. The entire expression cassette is cloned as a 2.5 kb EcoRI/SstI fragment into the binary vector pMOG23 linearized with EcoRI and SstI. The resulting binary vector, pMOG799 (Fig. 13), is used to

30

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- 30 -

transform potato (An E. coli strain harbouring pMOG799 has been deposited at the Centraal Bureau voor Schimmelcultures, Phabagen collections, Padualaan 8, Utrecht, The Netherlands, on August 23, 1993, deposit number CBS 430.93).

5

#### Example IX

##### Trehalose production in potatoes transformed with pMOG799

Potato tuber discs are transformed with the binary vector pMOG799 using Agrobacterium tumefaciens. Transgenic shoots  
10 are selected on kanamycin. A number of 20 independent transgenic shoots are analyzed for trehalose phosphate synthase (TPS) activity. Shoots found to contain the enzyme are grown to mature plants. Analyses of mature tubers of those transgenic potato plants show elevated levels of  
15 trehalose in comparison with non-transgenic control plants. Transgenic plant line MOG799.1 is propagated for further work.

#### Example X

##### Construction of pMOG664

20 Two oligonucleotides corresponding to the cDNA sequence of the small subunit of ADP-glucose pyrophosphorylase (AGPaseB) from potato tuber cv. Désirée (Müller-Röber, B., Kossmann, J., Hannah, L.C., Willmitzer, L., and Sonnewald, U. (1990) Mol. Gen. Genet. 224, 136-146) are synthesized:

25.

5' TCCCCATGGAATCAAAGCATCC 3' (SEQIDNO: 4)

5' GATTGGATCCAGGGCACGGCTG 3' (SEQIDNO: 5)

The oligonucleotides are designed to contain suitable  
30 restriction sites (BamHI and NcoI, underlined) at their termini to allow assembly in an expression cassette in an antisense orientation after digestion with these enzymes. A fragment of about 1 kb is PCR amplified with these oligonucleotides using DNA isolated from a cDNA library from  
35 potato cv. Désirée prepared from 2 month old leaf tissue (Clontech) as a template. By sequencing it is shown, that the fragment is identical with the AGPase B sequence from potato cv. Désirée (Müller-Röber, B., Kossmann, J., Hannah, L.C., Willmitzer, L., and Sonnewald, U. (1990) Mol. Gen. Genet.



- 31 -

224, 136-146). Following digestion with BamHI and NcoI, the fragment is cloned in pMOG18 linearized with BamHI and NcoI. From the resulting plasmid the 1.85 kb EcoRI/BamHI fragment (containing the CaMV 35S promoter, the AMV RNA4 leader and the AGPase fragment in an antisense orientation), as well as the BamHI/HindIII fragment containing the terminator from the nopaline synthase (NOS) gene from Agrobacterium tumefaciens are cloned in a three-way ligation in the binary vector pMOG22 linearized with EcoRI and HindIII. The binary vector pMOG22 contains a plant expressible HPTII gene for hygromycin selection in transgenic plants (pMOG22 has been deposited at the Centraal Bureau voor Schimmelcultures on January 29, 1990 under accession number 101.90). The resulting binary vector pMOG664 (Fig. 4) is used for potato transformation.

15

#### Example XI

##### Construction of pMOG801

A set of oligonucleotides complementary to the sequence of the maize sucrose phosphate synthase (SPS) cDNA (Worrell, A.C., Bruneau, J-M., Summerfalt, K., Boersig, M., and Voelker, T.A. (1991) Plant Cell 3, 1121) is synthesized. Their sequences are as follows:

5' CTAGGTCGTGATTCTGATACAGGTGGCCAGGTG 3' (SEQIDNO: 6)  
25 5' CAGCATCGGCATAGTGCCCATGTATCACGTAAGGC 3' (SEQIDNO: 7)

These oligonucleotides are used to PCR amplify a DNA fragment of 370 bp using DNA isolated from a potato cv. Désiree cDNA library prepared from 2 month old leaf tissue (Clontech) as a template. By sequencing of this fragment it is shown, that it is homologous to the SPS sequence of maize (see Figure 4, and Worrell et al. (1991). The PCR fragment is used to screen a lambda gt10 library of potato cv. Désiree cDNA library prepared from 2 month old leaf tissue (Clontech). The insert of one positively hybridizing clone is sequenced. The sequence of the 654 bp DNA fragment is found to be 65% identical with the corresponding part of the maize SPS sequence (Starting at nucleotide number 349 in Figure 11 in Worrell et al. (1991). The EcoRI insert of this clone is

- 32 -

cloned in pMOG180 digested with BamHI, in a three-way ligation with the following synthetic oligonucleotide duplex.

5' GATCGTCAGATCTAGC 3' (SEQIDNO: 14)

5 3' CAGTCTAGATCGTTAA 5' (SEQIDNO: 15)

The plasmid, having the SPS fragment in the antisense orientation with respect to the CaMV 35S promoter, is designated pMOG787. The EcoRI/HindIII fragment of plasmid  
10 pMOG787 is cloned in a three-way ligation with a synthetic linker:

5' AGCTTCCCCCCCCG 3' (SEQIDNO: 16)

3' AGGGGGGGCTTAA 5' (SEQIDNO: 17)

15

into the binary vector pMOG22 linearized with EcoRI. The binary vector pMOG22 contains a plant expressible HPTII gene for hygromycin selection in transgenic plants (pMOG22 has been deposited at the Centraal Bureau voor Schimmelcultures  
20 on January 29, 1990 under accession number 101.90). The resulting binary vector pMOG801 (Fig. 14) is used for potato transformation.

#### Example XII

25

##### Construction of pMOG802

The EcoRI fragment of plasmid pMOG801, containing the antisense SPS expression cassette, is cloned in the binary vector pMOG664 (containing the antisense AGPase cassette), linearized with EcoRI. The resulting binary vector is called  
30 pMOG802 (Fig 15).

#### Example XIII

##### Trehalose production in potato transformed with pMOG799 and pMOG664

35 Potato tuber discs of kanamycin resistant plant line MOG799.1, expressing TPS (Example IX) are transformed with the binary vector pMOG664, containing the antisense AGPase expression cassette. Transgenic shoots, selected on 10 mg/L hygromycin, are analyzed for the presence of the TPS and

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antisense AGPase sequences by PCR. Transgenic plants containing both are analyzed for TPS and AGPase activity. By analysis of transgenic tubers for AGPase activity it is shown that, reductions in activity levels in individual  
5 transgenic lines in comparison with non-transgenic controls occur. By Northern blots it is shown, that mRNA levels for AGPase are reduced in the transgenic plants compared to those in non-transgenic control plants. Trehalose levels in tubers of transgenic potato plants, found to exhibit TPS activity,  
10 and having reduced levels of AGPase, show an increase in comparison with the levels found in tubers of transgenic plant line MOG799.1.

#### Example XIV

15 Trehalose production in potato transformed with pMOG799 and pMOG801

Potato tuber discs of kanamycin resistant plant line MOG799.1, expressing TPS (Example IX) are transformed with the binary vector pMOG801, containing the antisense SPS  
20 expression cassette. Transgenic shoots, selected on 10 mg/L hygromycin, are analyzed for the presence of the TPS and antisense SPS sequences by PCR. Transgenic plants containing both are analyzed for TPS and SPS activity. By analysis of transgenic tubers for SPS activity it is shown  
25 that reductions in activity levels in individual transgenic lines in comparison with non-transgenic controls occur. By Northern blots it is shown, that mRNA levels for SPS are reduced in the transgenic plants compared to those in non-transgenic control plants. Trehalose levels in tubers of  
30 transgenic potato plants, found to exhibit TPS activity, and having reduced levels of SPS, show an increase in comparison with the levels found in tubers of transgenic plant line MOG799.1.

35

#### Example XV

Trehalose production in potato transformed with pMOG799 and pMOG802

Potato tuber discs of kanamycin resistant plant line MOG799.1, expressing TPS (Example IX) are transformed with

- 34 -

the binary vector pMOG802, containing the antisense SPS and AGPase expression cassettes. Transgenic shoots, selected on 10 mg/L hygromycin, are analyzed for the presence of the TPS, antisense AGPase and antisense SPS sequences by PCR.

- 5 Transgenic plants containing all three constructs are analyzed for TPS, AGPase and SPS activity.

By analysis of transgenic tubers for AGPase and SPS activity it is shown, that reductions in the activity levels for both enzymes in individual transgenic lines in comparison with  
10 non-transgenic controls occur. By Northern blots it is shown that mRNA levels for AGPase and SPS are reduced in the transgenic plants compared to those in non-transgenic control plants. Trehalose levels in tubers of transgenic potato plants, found to exhibit TPS activity, and having reduced  
15 levels of SPS, show an increase in comparison with the levels found in tubers of transgenic plant line MOG799.1.

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

5

## (i) APPLICANT:

- (A) NAME: MOGEN International N.V.
- (B) STREET: Einsteinweg 97
- (C) CITY: LEIDEN
- (D) STATE: Zuid-Holland
- (E) COUNTRY: The Netherlands
- (F) POSTAL CODE (ZIP): NL-2333 CB
- (G) TELEPHONE: (31).(71).258282
- (H) TELEFAX: (31).(71).221471

15

(ii) TITLE OF INVENTION: PRODUCTION OF TREHALOSE IN PLANTS

(iii) NUMBER OF SEQUENCES: 17

20

## (iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

25

## (v) CURRENT APPLICATION DATA:

APPLICATION NUMBER: WO PCT/EP93/02290

## (2) INFORMATION FOR SEQ ID NO: 1:

30

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 370 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

40

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: Solanum tuberosum
- (B) STRAIN: Desiree
- (F) TISSUE TYPE: Leaf

45

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CTAGGTGCTG ATTCTGATAC AGGTGGCCAG GTGAAGTATG TAGTAGAGCT TGCTGAGCA	60
CTTGCAAACA TGAAAGGAGT TCACCGAGTT GATCTCTTGA CTGGCAGAT CACATCCCA	120
GAGGTGATT CTAGCTATGG TGAGCCAATT GAGATGCTCT CATGCCATC TGATGCTTTG	180
GCTGCTGTGG TGCTACTAT TGGATOCCT GGGACAGG TGACAAGATA TTCCAAAAGA	240

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ATTACATAC CAGAATTGT TGATGGAGCA TTAAGOCACA TTGTGAATAT GGCAAGGGCT 300  
 ATAGGGGAGC AAGTCAATGC TGGAAAAGCA GTGTGGCCTT ACGTGATACA TGGGCACTAT 360  
 5 GCOGATGCTG 370

## (2) INFORMATION FOR SEQ ID NO: 2:

- (i) SEQUENCE CHARACTERISTICS:  
 10 (A) LENGTH: 1446 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)  
 15 (iii) HYPOTHETICAL: NO
- (vi) ORIGINAL SOURCE:  
 20 (A) ORGANISM: Escherichia coli
- (vii) IMMEDIATE SOURCE:  
 (B) CLONE: 7F11
- (viii) POSITION IN GENOME:  
 25 (B) MAP POSITION: 41-42'
- (ix) FEATURE:  
 30 (A) NAME/KEY: CDS  
 (B) LOCATION: 19..1446  
 (D) OTHER INFORMATION: /product= "trehalose phosphate  
 synthase"  
 /gene= "otsA"

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GAGAAAATAA CAGGAGTG ATG ACT ATG AGT GGT TTA GTC GTA GTA TCT AAC 51  
 Met Thr Met Ser Arg Leu Val Val Val Ser Asn  
 40 1 5 10

CGG ATT GCA CCA CCA GAC GAG CAC GCC GCC AGT GCC GGT GGC CTT GCC 99  
 Arg Ile Ala Pro Pro Asp Glu His Ala Ala Ser Ala Gly Gly Leu Ala  
 15 20 25

GTT GGC ATA CTG GGG GCA CTG AAA GCC GCA GGC GGA CTG TGG TTT GGC 147  
 Val Gly Ile Leu Gly Ala Leu Lys Ala Ala Gly Gly Leu Trp Phe Gly  
 30 35 40

TGG AGT GGT GAA ACA GGG AAT GAG GAT CAG CCG CTA AAA AAG GTG AAA 195  
 Trp Ser Gly Glu Thr Gly Asn Glu Asp Gln Pro Leu Lys Lys Val Lys  
 45 50 55

AAA GGT AAC ATT ACG TGG GCC TCT TTT AAC CTC AGC GAA CAG GAC CTT 243  
 55 Lys Gly Asn Ile Thr Trp Ala Ser Phe Asn Leu Ser Glu Gln Asp Leu  
 60 65 70 75

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	GAC GAA TAC TAC AAC CAA TTC TCC AAT GGC GTT CTC TGG CCC GCT TTT	291
	Asp Glu Tyr Tyr Asn Gln Phe Ser Asn Ala Val Leu Trp Pro Ala Phe	
	80 85 90	
5	CAT TAT CGG CTC GAT CTG GTG CAA TTT CAG CGT OCT GGC TGG GAC GGC	339
	His Tyr Arg Leu Asp Leu Val Gln Phe Gln Arg Pro Ala Trp Asp Gly	
	95 100 105	
10	TAT CTA CGC GTA AAT GCG TTG CTG GCA GAT AAA TTA CTG CCG CTG TTG	387
	Tyr Leu Arg Val Asn Ala Leu Leu Ala Asp Lys Leu Leu Pro Leu Leu	
	110 115 120	
15	CAA GAC GAT GAC ATT ATC TGG ATC CAC GAT TAT CAC CTG TTG CCA TTT	435
	Gln Asp Asp Asp Ile Ile Trp Ile His Asp Tyr His Leu Leu Pro Phe	
	125 130 135	
20	GCG CAT GAA TTA CGC AAA CGG GGA GTG AAT AAT CGC ATT GGT TTC TTT	483
	Ala His Glu Leu Arg Lys Arg Gly Val Asn Asn Arg Ile Gly Phe Phe	
	140 145 150 155	
	CTG CAT ATT OCT TTC CCG ACA CCG GAA ATC TTC AAC GCG CTG CCG ACA	531
	Leu His Ile Pro Phe Pro Thr Pro Glu Ile Phe Asn Ala Leu Pro Thr	
	160 165 170	
25	TAT GAC ACC TTG CTT GAA CAG CTT TGT GAT TAT GAT TTG CTG GGT TTC	579
	Tyr Asp Thr Leu Leu Glu Gln Leu Cys Asp Tyr Asp Leu Leu Gly Phe	
	175 180 185	
30	CAG ACA GAA AAC GAT CGT CTG GCG TTC CTG GAT TGT CTT TCT AAC CTG	627
	Gln Thr Glu Asn Asp Arg Leu Ala Phe Leu Asp Cys Leu Ser Asn Leu	
	190 195 200	
35	ACC CGC GTC ACG ACA CGT AGC GCA AAA AGC CAT ACA GGC TGG GGC AAA	675
	Thr Arg Val Thr Thr Arg Ser Ala Lys Ser His Thr Ala Trp Gly Lys	
	205 210 215	
40	GCA TTT CGA ACA GAA GTC TAC CCG ATC GGC ATT GAA CCG AAA GAA ATA	723
	Ala Phe Arg Thr Glu Val Tyr Pro Ile Gly Ile Glu Pro Lys Glu Ile	
	220 225 230 235	
	GCC AAA CAG GCT GCG GGG CCA CTG CCG CCA AAA CTG GCG CAA CTT AAA	771
	Ala Lys Gln Ala Ala Gly Pro Leu Pro Pro Lys Leu Ala Gln Leu Lys	
	240 245 250	
45	GCG GAA CTG AAA AAC GTA CAA AAT ATC TTT TCT GTC GAA CCG CTG GAT	819
	Ala Glu Leu Lys Asn Val Gln Asn Ile Phe Ser Val Glu Arg Leu Asp	
	255 260 265	
50	TAT TCC AAA GGT TTG CCA GAG CGT TTT CTC GGC TAT GAA GCG TTG CTG	867
	Tyr Ser Lys Gly Leu Pro Glu Arg Phe Leu Ala Tyr Glu Ala Leu Leu	
	270 275 280	
55	GAA AAA TAT CCG CAG CAT CAT GGT AAA ATT CGT TAT ACC CAG ATT GCA	915
	Glu Lys Tyr Pro Gln His His Gly Lys Ile Arg Tyr Thr Gln Ile Ala	
	285 290 295	

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	CCA	ACG	TOG	OGT	GGT	GAT	GTG	CAA	GCC	TAT	CAG	GAT	ATT	OGT	CAT	CAG	963
	Pro	Thr	Ser	Arg	Gly	Asp	Val	Gln	Ala	Tyr	Gln	Asp	Ile	Arg	His	Gln	
	300					305					310					315	
5	CTC	GAA	AAT	GAA	GCT	GGA	CGA	ATT	AAT	GGT	AAA	TAC	GGG	CAA	TTA	GGC	1011
	Leu	Glu	Asn	Glu	Ala	Gly	Arg	Ile	Asn	Gly	Lys	Tyr	Gly	Gln	Leu	Gly	
					320					325						330	
10	TGG	ACG	COG	CTT	TAT	TAT	TIG	AAT	CAG	CAT	TTT	GAC	OGT	AAA	TTA	CTG	1059
	Trp	Thr	Pro	Leu	Tyr	Tyr	Leu	Asn	Gln	His	Phe	Asp	Arg	Lys	Leu	Leu	
				335					340					345			
15	ATG	AAA	ATA	TTC	OGC	TAC	TCT	GAC	GTG	GGC	TTA	GTG	ACG	CCA	CTG	OGT	1107
	Met	Lys	Ile	Phe	Arg	Tyr	Ser	Asp	Val	Gly	Leu	Val	Thr	Pro	Leu	Arg	
			350					355					360				
20	GAC	GGG	ATG	AAC	CTG	GTA	GCA	AAA	GAG	TAT	GTT	GCT	GCT	CAG	GAC	CCA	1155
	Asp	Gly	Met	Asn	Leu	Val	Ala	Lys	Glu	Tyr	Val	Ala	Ala	Gln	Asp	Pro	
		365					370					375					
	GOC	AAT	COG	GGC	GTT	CTT	GTT	CTT	TOG	CAA	TTT	GCG	GGA	GCG	GCA	AAC	1203
	Ala	Asn	Pro	Gly	Val	Leu	Val	Leu	Ser	Gln	Phe	Ala	Gly	Ala	Ala	Asn	
	380					385					390					395	
25	GAG	TTA	ACG	TOG	GCG	TTA	ATT	GTT	AAC	CCC	TAC	GAT	OGT	GAC	GAA	GTT	1251
	Glu	Leu	Thr	Ser	Ala	Leu	Ile	Val	Asn	Pro	Tyr	Asp	Arg	Asp	Glu	Val	
					400					405					410		
30	GCA	GCT	GCG	CTG	GAT	OGT	GCA	TIG	ACT	ATG	TOG	CTG	GCG	GAA	OGT	ATT	1299
	Ala	Ala	Ala	Leu	Asp	Arg	Ala	Leu	Thr	Met	Ser	Leu	Ala	Glu	Arg	Ile	
				415					420					425			
35	TCC	OGT	CAT	GCA	GAA	ATG	CTG	GAC	GTT	ATC	GTG	AAA	AAC	GAT	ATT	AAC	1347
	Ser	Arg	His	Ala	Glu	Met	Leu	Asp	Val	Ile	Val	Lys	Asn	Asp	Ile	Asn	
			430					435					440				
40	CAC	TGG	CAG	GAG	TGC	TTC	ATT	AGC	GAC	CTA	AAG	CAG	ATA	GTT	COG	CGA	1395
	His	Trp	Gln	Glu	Cys	Phe	Ile	Ser	Asp	Leu	Lys	Gln	Ile	Val	Pro	Arg	
		445					450					455					
	AGC	GCG	GAA	AGC	CAG	CAG	CGC	GAT	AAA	GTT	GCT	ACC	TTT	CCA	AAG	CTT	1443
	Ser	Ala	Glu	Ser	Gln	Gln	Arg	Asp	Lys	Val	Ala	Thr	Phe	Pro	Lys	Leu	
	460					465					470					475	
45	GCG																1446
	Ala																

50 (2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 476 amino acids

(B) TYPE: amino acid

55 (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

5 Met Thr Met Ser Arg Leu Val Val Val Ser Asn Arg Ile Ala Pro Pro  
 1 5 10 15  
 Asp Glu His Ala Ala Ser Ala Gly Gly Leu Ala Val Gly Ile Leu Gly  
 20 25 30  
 10 Ala Leu Lys Ala Ala Gly Gly Leu Trp Phe Gly Trp Ser Gly Glu Thr  
 35 40 45  
 Gly Asn Glu Asp Gln Pro Leu Lys Lys Val Lys Lys Gly Asn Ile Thr  
 15 50 55 60  
 Trp Ala Ser Phe Asn Leu Ser Glu Gln Asp Leu Asp Glu Tyr Tyr Asn  
 65 70 75 80  
 20 Gln Phe Ser Asn Ala Val Leu Trp Pro Ala Phe His Tyr Arg Leu Asp  
 85 90 95  
 Leu Val Gln Phe Gln Arg Pro Ala Trp Asp Gly Tyr Leu Arg Val Asn  
 100 105 110  
 25 Ala Leu Leu Ala Asp Lys Leu Leu Pro Leu Leu Gln Asp Asp Ile  
 115 120 125  
 Ile Trp Ile His Asp Tyr His Leu Leu Pro Phe Ala His Glu Leu Arg  
 130 135 140  
 Lys Arg Gly Val Asn Asn Arg Ile Gly Phe Phe Leu His Ile Pro Phe  
 145 150 155 160  
 35 Pro Thr Pro Glu Ile Phe Asn Ala Leu Pro Thr Tyr Asp Thr Leu Leu  
 165 170 175  
 Glu Gln Leu Cys Asp Tyr Asp Leu Leu Gly Phe Gln Thr Glu Asn Asp  
 180 185 190  
 40 Arg Leu Ala Phe Leu Asp Cys Leu Ser Asn Leu Thr Arg Val Thr Thr  
 195 200 205  
 Arg Ser Ala Lys Ser His Thr Ala Trp Gly Lys Ala Phe Arg Thr Glu  
 210 215 220  
 Val Tyr Pro Ile Gly Ile Glu Pro Lys Glu Ile Ala Lys Gln Ala Ala  
 225 230 235 240  
 50 Gly Pro Leu Pro Pro Lys Leu Ala Gln Leu Lys Ala Glu Leu Lys Asn  
 245 250 255  
 Val Gln Asn Ile Phe Ser Val Glu Arg Leu Asp Tyr Ser Lys Gly Leu  
 260 265 270  
 55 Pro Glu Arg Phe Leu Ala Tyr Glu Ala Leu Leu Glu Lys Tyr Pro Gln

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	275	280	285
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5	Asp Val Gln Ala Tyr Gln Asp Ile Arg His Gln Leu Glu Asn Glu Ala		
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	Gly Arg Ile Asn Gly Lys Tyr Gly Gln Leu Gly Trp Thr Pro Leu Tyr		
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	Tyr Leu Asn Gln His Phe Asp Arg Lys Leu Leu Met Lys Ile Phe Arg		
		340	345 350
15	Tyr Ser Asp Val Gly Leu Val Thr Pro Leu Arg Asp Gly Met Asn Leu		
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20	Leu Val Leu Ser Gln Phe Ala Gly Ala Ala Asn Glu Leu Thr Ser Ala		
		385	390 395 400
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25		405	410 415
	Arg Ala Leu Thr Met Ser Leu Ala Glu Arg Ile Ser Arg His Ala Glu		
		420	425 430
30	Met Leu Asp Val Ile Val Lys Asn Asp Ile Asn His Trp Gln Glu Cys		
		435	440 445
	Phe Ile Ser Asp Leu Lys Gln Ile Val Pro Arg Ser Ala Glu Ser Gln		
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35	Gln Arg Asp Lys Val Ala Thr Phe Pro Lys Leu Ala		
		465	470 475

## (2) INFORMATION FOR SEQ ID NO: 4:

- 40 (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 22 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - 45 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: YES

50

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

55 TCCCCATGGA ATCAAAGCAT CC

22

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## (2) INFORMATION FOR SEQ ID NO: 5:

## (i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 22 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

10

## (iii) HYPOTHETICAL: YES

## 15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GATTGGATOC AGGGCAOGGC TG

22

## (2) INFORMATION FOR SEQ ID NO: 6:

20

## (i) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 33 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (iii) HYPOTHETICAL: YES

30

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

35 CTAGGTGCTG ATTCTGATAC AGGTGGCCAG GTG

33

## (2) INFORMATION FOR SEQ ID NO: 7:

## (i) SEQUENCE CHARACTERISTICS:

- 40 (A) LENGTH: 35 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

45

## (iii) HYPOTHETICAL: YES

50

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

CAGCATCGGC ATAGTGCCCA TGTATCAOCT AAGGC

35

## 55 (2) INFORMATION FOR SEQ ID NO: 8:

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- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 18 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
5      (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: YES  
10
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:
- 15 AGCTCAGGAG CTCTCAGG 18
- (2) INFORMATION FOR SEQ ID NO: 9:
- (i) SEQUENCE CHARACTERISTICS:  
20      (A) LENGTH: 18 base pairs  
        (B) TYPE: nucleic acid  
        (C) STRANDEDNESS: single  
        (D) TOPOLOGY: linear
- 25      (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: YES
- 30
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:
- GTGCTGAGAG GTCTCTGA 18
- 35 (2) INFORMATION FOR SEQ ID NO: 10:
- (i) SEQUENCE CHARACTERISTICS:  
        (A) LENGTH: 24 base pairs  
        (B) TYPE: nucleic acid  
40      (C) STRANDEDNESS: single  
        (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- 45      (iii) HYPOTHETICAL: YES
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:
- 50 GATCCCCCGG GGCATGCAAG CTTG 24
- (2) INFORMATION FOR SEQ ID NO: 11:
- 55      (i) SEQUENCE CHARACTERISTICS:  
        (A) LENGTH: 24 base pairs

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(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: YES

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GGGGCCCCGT ACGTTOGAAC CTAG

24

15 (2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 base pairs

(B) TYPE: nucleic acid

20 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

25 (iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

30

GAGAAAATAC CCGGGGIGAT GAC

23

(2) INFORMATION FOR SEQ ID NO: 13:

35

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

40

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: YES

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

GATAATOGTG GATCCAGATA ATGTC

25

50

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 base pairs

55 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single

SUBSTITUTE SHEET

- 44 -

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

5 (iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

10

GATOGTCAGA TCTAGC

16

(2) INFORMATION FOR SEQ ID NO: 15:

15

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: YES

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

CAGTCTAGAT CGTTAA

16

30

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 13 base pairs

35

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

40

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: YES

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

AGCTTCCCCC CCG

13

50

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 13 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

55

(D) TOPOLOGY: linear

- 45 -

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: YES

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

AGGGGGGGCT TAA

13

10

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## CLAIMS

1. A plant expressible gene which when expressed in a plant or plant cell increases the trehalose content of said plant or plant cell.
- 5 2. A plant expressible gene according to claim 1 which comprises in sequence:
  - (a) a transcriptional initiation region that is functional in said plant host,
  - 10 (b) a DNA sequence encoding a trehalose phosphate synthase activity, and optionally
  - (c) a transcriptional termination sequence that is functional in said plant host.
- 15 3. A DNA sequence containing a plant expressible gene which comprises in sequence:
  - (a) a transcriptional initiation region that is functional in said plant host,
  - (b) a DNA sequence encoding a trehalose phosphate synthase
  - 20 activity, and optionally
  - (c) a transcriptional termination sequence that is functional in said plant host,
  - and a plant expressible gene comprising in sequence:
    - (a) a transcriptional initiation region that is functional in
    - 25 said plant host,
    - (b) a DNA sequence encoding an RNA sequence at least partially complementary to an RNA sequence which encodes sucrose phosphate synthase enzyme (SPS) naturally occurring in said plant host, and optionally
    - 30 (c) a transcriptional termination sequence that is functional in said plant host.
4. A DNA sequence comprising a plant expressible gene which comprises in sequence:
  - 35 (a) a transcriptional initiation region that is functional in said plant host,
  - (b) a DNA sequence encoding a trehalose phosphate synthase



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activity, and optionally

(c) a transcriptional termination sequence that is functional in said plant host, and

a plant expressible gene comprising in sequence:

5 (a) a transcriptional initiation region that is functional in said plant host,

(b) a DNA sequence encoding an RNA sequence at least partially complementary to an RNA sequence which encodes a ADP-glucose pyrophosphorylase enzyme naturally occurring in

10 said plant host, and optionally

(c) a transcriptional termination sequence that is functional in said plant host.

5. A DNA sequence comprising a plant expressible  
15 gene which comprises in sequence:

(a) a transcriptional initiation region that is functional in said plant host,

(b) a DNA sequence encoding a trehalose phosphate synthase activity, and optionally

20 (c) a transcriptional termination sequence that is functional in said plant host,

and a plant expressible gene comprising in sequence:

(a) a transcriptional initiation region that is functional in said plant host,

25 (b) a DNA sequence encoding an RNA sequence at least partially complementary to an RNA sequence which encodes a sucrose phosphate synthase enzyme naturally occurring in said plant host, and optionally

(c) a transcriptional termination sequence that is functional  
30 in said plant host,

and a plant expressible gene comprising in sequence:

(a) a transcriptional initiation region that is functional in said plant host,

35 (b) a DNA sequence encoding an RNA sequence at least partially complementary to an RNA sequence which encodes a ADP-glucose pyrophosphorylase enzyme naturally occurring in said plant host, and optionally

- 48 -

(c) a transcriptional termination sequence that is functional in said plant host.

6. A vector suitable for cloning which comprises a plant expressible gene according to claim 1 or 2.

7. A vector suitable for cloning which comprises a DNA sequence of any one of the claims 3 to 5.

8. A vector according to claim 6 or 7 which is a binary vector.

9. A microorganism comprising a vector of any one of the claims 6 to 8.

15

10. The microorganism of claim 9 which is of the genus Agrobacterium.

11. A method for obtaining a plant capable of producing trehalose comprising the steps of,

(1) introducing into a recipient cell of a plant a plant expressible gene which when expressed in a plant or plant cell increases the trehalose content of said plant or plant cell,

25 and a plant expressible gene comprising in sequence:

(a) a transcriptional initiation region that is functional in said plant host,

(b) a DNA sequence encoding a selectable marker gene that is functional in said plant host, and optionally

30 (c) a transcriptional termination sequence that is functional in said plant host,

(2) generating a plant from a transformed cell under conditions that allow for selection for the presence of the selectable marker gene.

35

12. A recombinant plant DNA genome which contains a plant expressible trehalose phosphate synthase gene that is

- 49 -

not naturally present therein.

13. A recombinant plant DNA genome which comprises  
(a) a plant expressible gene encoding trehalose phosphate  
5 synthase, and  
(b) a plant expressible gene capable of inhibiting the  
biosynthesis of a sucrose phosphate synthesis activity.
14. A recombinant plant DNA genome which comprises:  
10 (a) a plant expressible gene encoding trehalose phosphate  
synthase,  
(b) a plant expressible gene capable of inhibiting the  
biosynthesis of an ADP-Glucose pyrophosphorylase activity.
- 15 15. A recombinant plant DNA genome which comprises:  
(a) a plant expressible gene encoding trehalose phosphate  
synthase,  
(b) a plant expressible gene capable of inhibiting the  
biosynthesis of an ADP-Glucose pyrophosphorylase activity,  
20 and  
(c) a plant expressible gene capable of inhibiting the  
biosynthesis of an sucrose phosphate synthesis activity.
16. A plant cell having a recombinant plant DNA genome  
25 of any one of the claims 12 to 15.
17. The plant cell of claim 16 which contains increased  
levels of trehalose compared with a plant cell of the same  
species having a non-recombinant plant DNA genome.  
30
18. A plant cell culture comprising plant cells of any  
one of the claims 16 or 17.
19. A method for the production of trehalose comprising  
35 the steps of:  
(1) growing in culture plant cells which by virtue of a  
recombinant plant DNA genome are capable of producing

- 50 -

(increased levels of) trehalose,  
(2) isolating the trehalose from the said plant cell culture.

20. The method of claim 19 wherein the plant cell  
5 culture is that of claim 18.

21. A plant containing a cell of any one of the claims  
16 to 17.

10 22. A plant consisting predominantly of cells of any one  
of the claims 16 to 17.

23. A plant capable of producing increased levels of  
trehalose as a result of genetic modification.

15

24. A plant having a recombinant plant DNA genome of any  
one of the claims 13 to 15.

25. The plant of any one of the claims 23 to 24 which  
20 contains increased levels of trehalose.

26. The plant of claim 25 which is belongs to the  
Angiospermae.

25 27. A part of a plant containing a cell of any one of  
the claims 16 to 17.

28. A part of a plant consisting predominantly of a cell  
of any one of the claims 16 or 17.

30

29. A part of a plant obtained from a plant of any one  
of the claims 22 to 25 wherein said part contains increased  
levels of trehalose.

35 30. A part according to any one of the claims 27 to 29  
selected from the group consisting of bulbs, flowers, fruits,  
hairy roots, leaves, microtubers, pollen, roots, seeds,

- 51 -

stalks and tubers.

31. A method of preserving a plant or plant part in the presence of trehalose, comprising the steps of:

- 5 (1) growing a plant of any one of the claims 25 to 26, or growing a plant part of any one of the claims 29 to 30,  
(2) harvesting the plant or the plant part which contains trehalose, and  
(3) air drying the plant or plant part or alternatively,  
10 (4) freeze drying the plant or plant part.

32. A dried plant or plant part which obtainable by the method of claim 31.

15 33. A method for the production of trehalose comprising the steps of:

- (1) growing a plant of claim 23 under conditions allowing for the production of trehalose,  
(2) harvesting said plant or a part thereof,  
20 (3) isolating the trehalose from the said plant or the said part thereof.

34. Trehalose which is substantially free from bacterial or yeast contaminants.

25

35. An isolated DNA sequence encoding a trehalose phosphate synthase activity.

36. An isolated DNA sequence according to claim 34,  
30 which is obtained from E. coli.

37. An isolated DNA sequence according to claim 35 which is represented by SEQIDNO: 2, or an isolated DNA sequence hybridising therewith under stringent conditions.

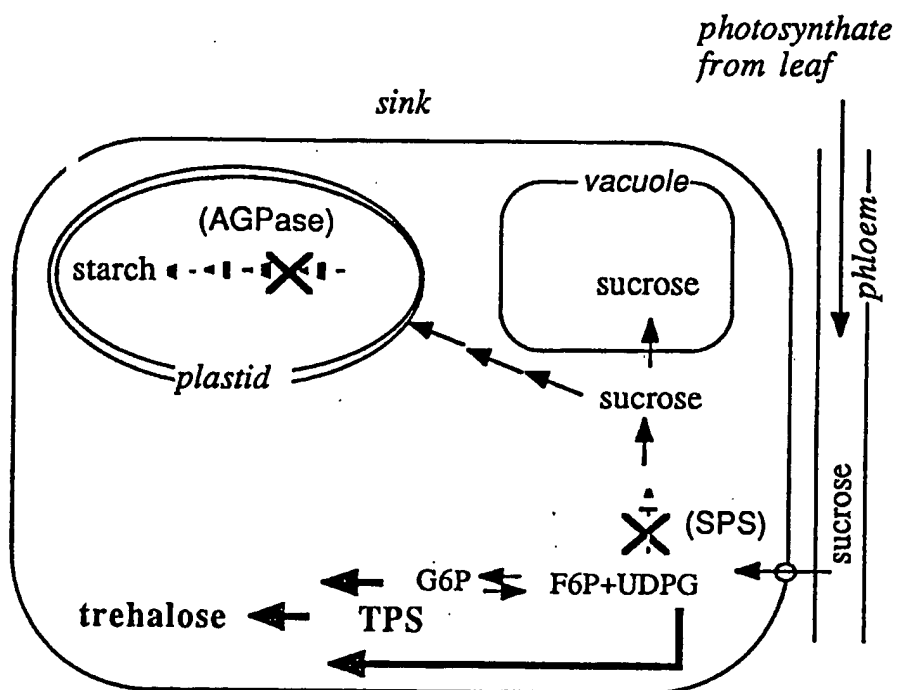
35

38. An isolated nucleic acid sequence that codes for the amino acid sequence of SEQIDNO: 3.

FIGURE 1.

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# ENGINEERING OF TREHALOSE-PRODUCTION IN PLANTS



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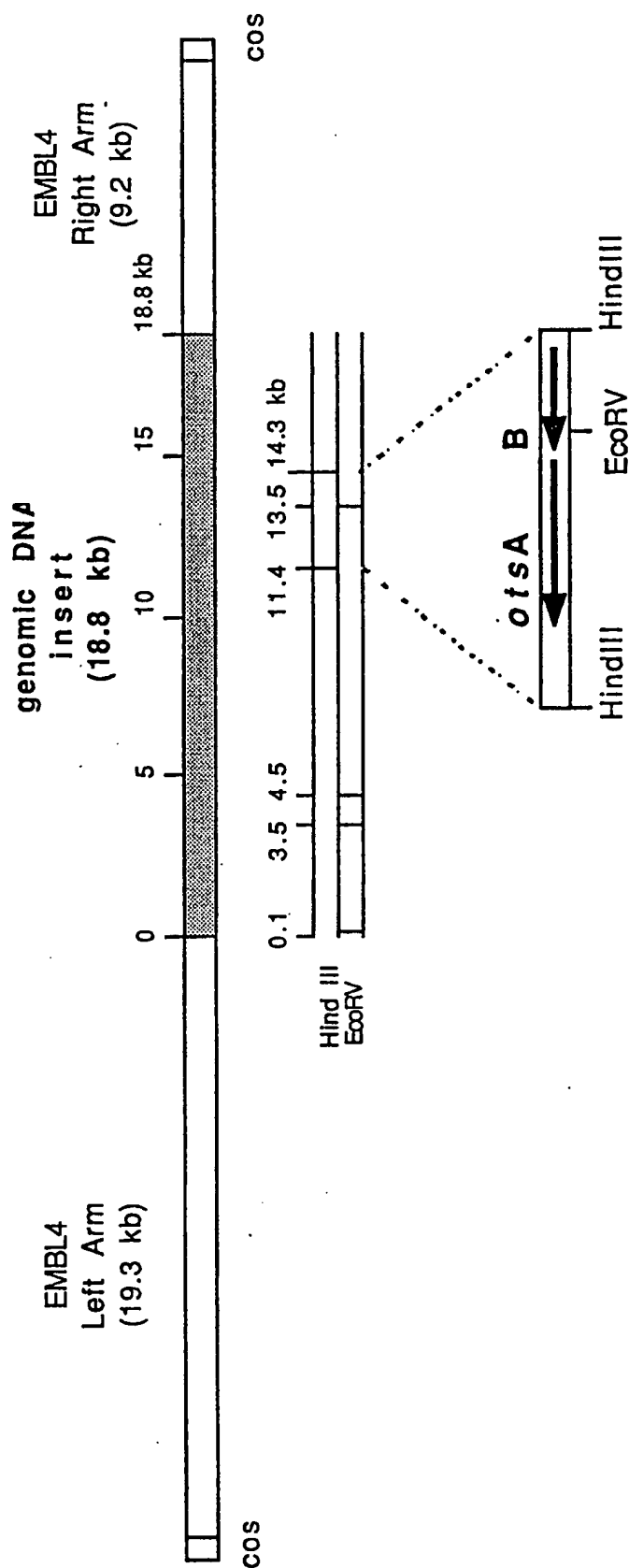


Figure 2

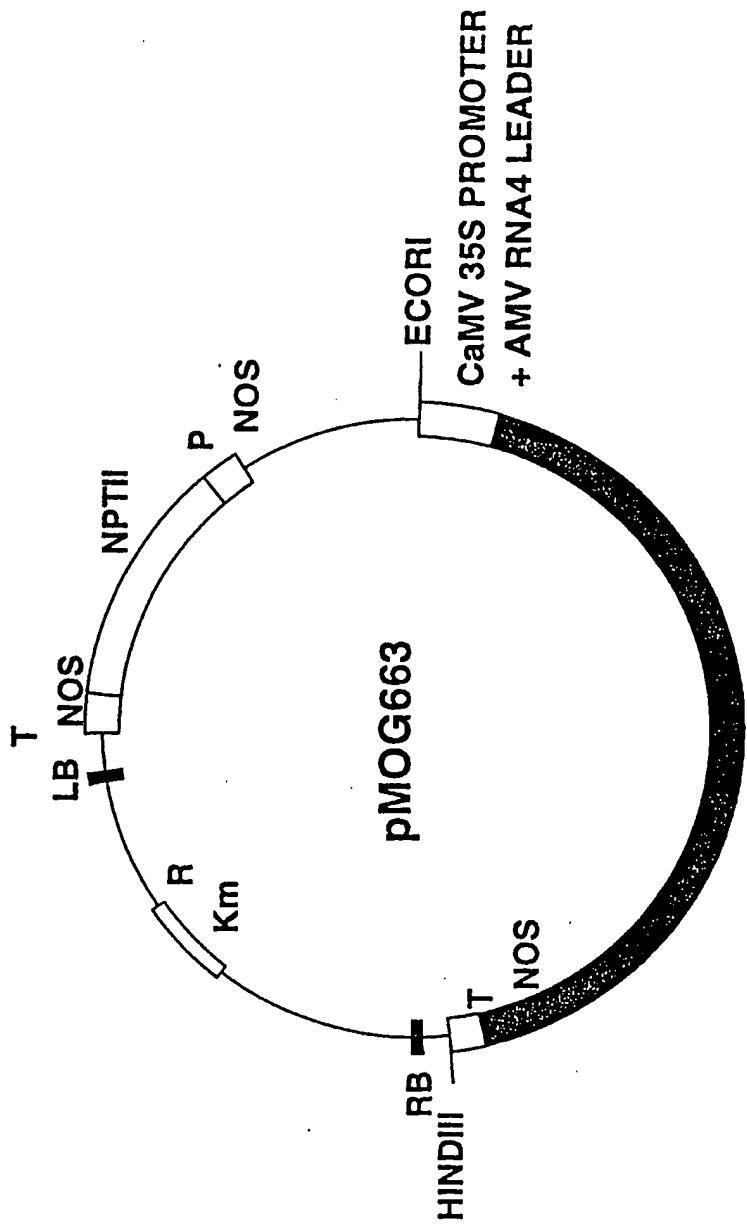


Figure 3

TREHALOSE-6-PHOSPHATE SYNTHASE



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1	CTAGGTCGTG	ATTCTGATAC	AGGTGGCCAG	GTGAAGTATG	TAGTAGAGCT	TGCTCGAGCA	60
61	CTTGCAAACA	TGAAAGGAGT	TCACCGAGTT	GATCTCTTGA	CTCGGCAGAT	CACATCCCCA	120
121	GAGGTTGATT	CTAGCTATGG	TGAGCCCAATT	GAGATGCTCT	CATGCCCATC	TGATGCTTTG	180
181	GCTGCTGTGG	TGCCCTACTAT	TCGGATCCCT	GCGGACCAGG	TGACAAAGATA	TTCCAAAAGA	240
241	ATTACATAC	CAGAAATTTGT	TGATGGAGCA	TTAAGCCACA	TTGTGAATAT	GGCAAGGGCT	300
301	ATAGGGGAGC	AAGTCAATGC	TGGA AAAAGCA	GTGTGGCCTT	ACGTGATACA	TGGC CACTAT	360
361	GCCGATGCTG						370

Figure 4

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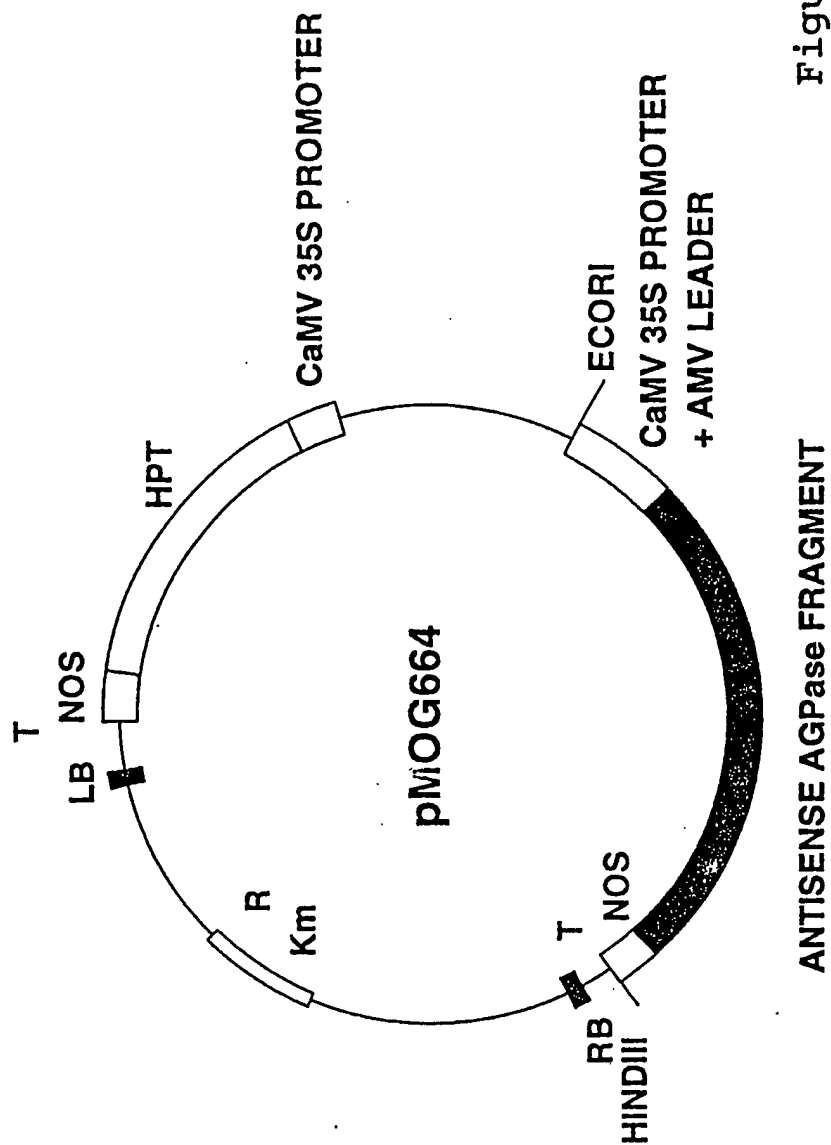


Figure 5

ANTISENSE AGPase FRAGMENT

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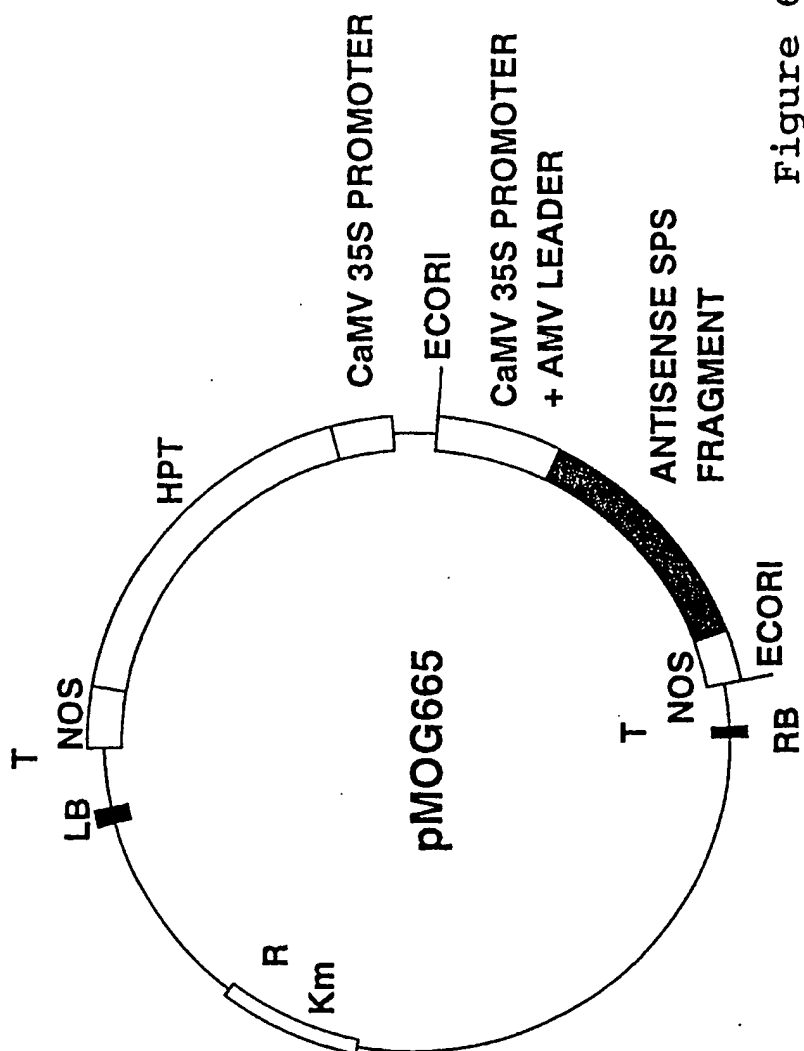


Figure 6

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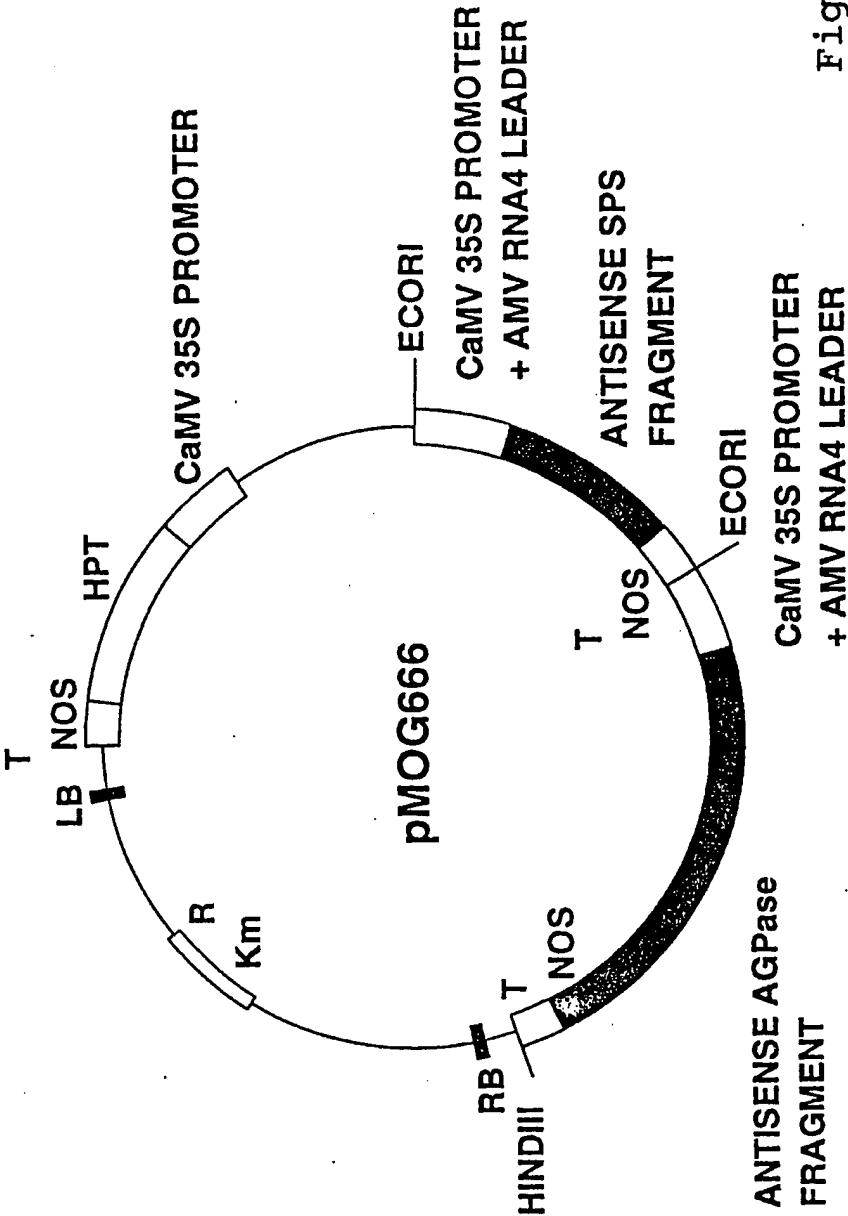


Figure 7

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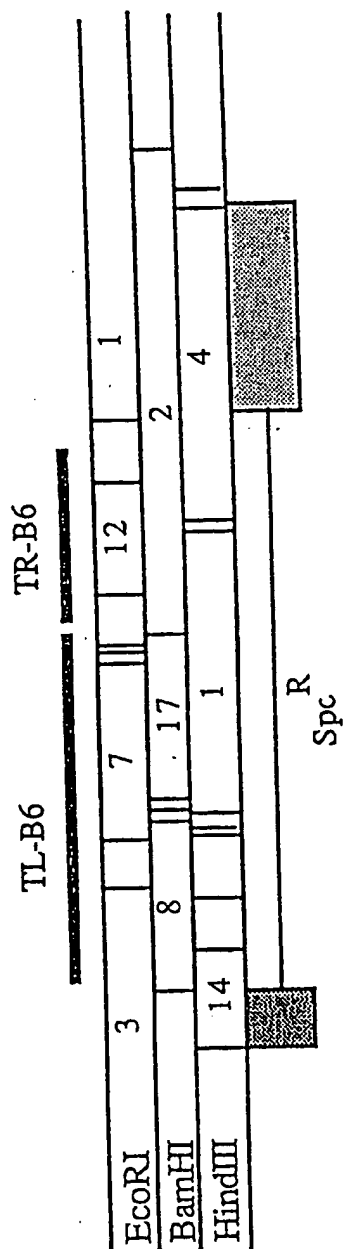


Figure 8

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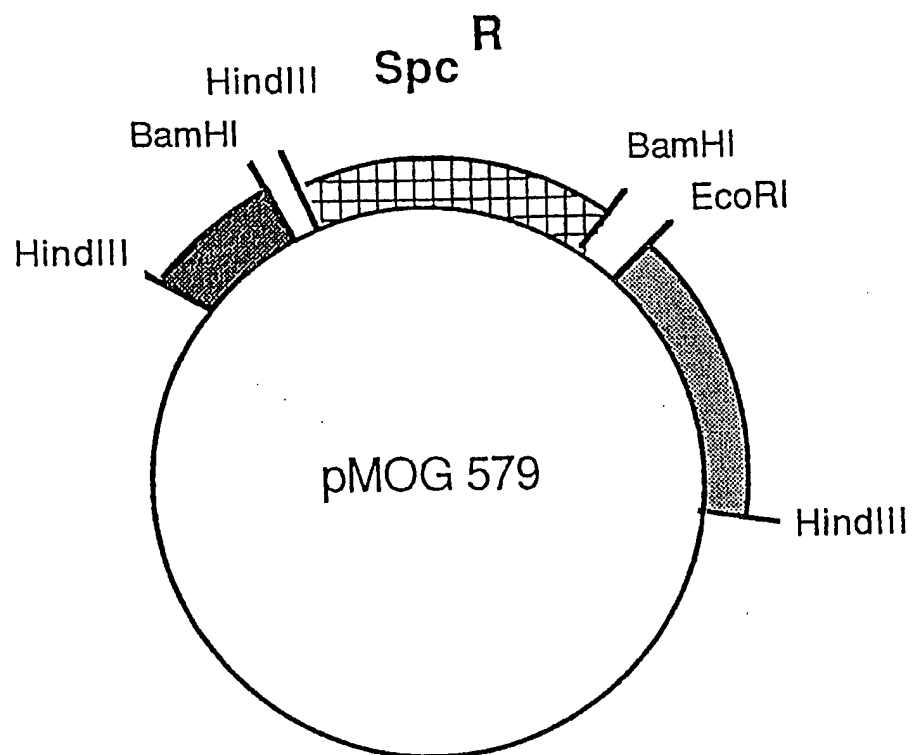


Figure 9

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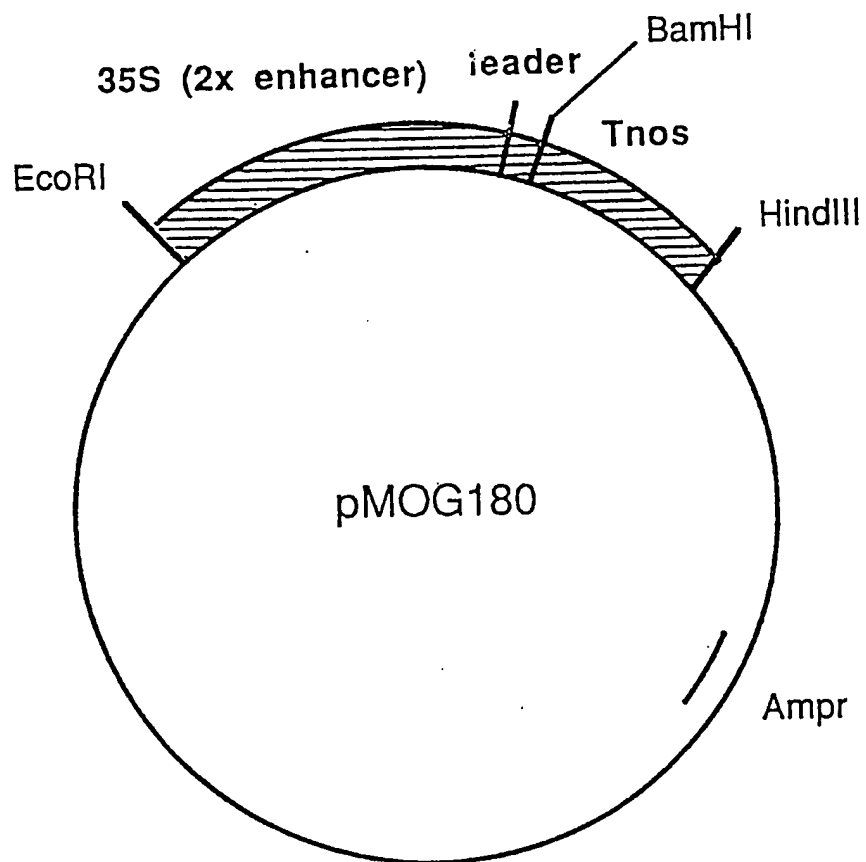


Figure 10

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-18/1  
 GAG AAA ATA ACA GGA GTG ATG ACT ATG AGT CGT TTA GTC GTA TCT AAC CGG ATT GCA  
 met thr met ser arg leu val val val ser asn arg ile ala  
 13/11  
 43/21  
 CCA CCA GAC GAG CAC GCC AGT GCC GGT GGC CTT GCC GTT GGC ATA CTG GGG GCA CTG  
 pro pro asp glu his ala ala ser ala gly gly leu ala val gly ile leu gly ala leu  
 103/41  
 AAA GCC GCA GCC GGA CTG TGG TTT GGC TGG AGT GGT GAA ACA GGG AAT GAG GAT CAG CCG  
 lys ala ala gly gly leu trp phe gly trp ser gly glu thr gly asn glu asp gln pro  
 163/61  
 CTA AAA AAG GTG AAA AAA GGT AAC ATT ACG TGG GCC TCT TTT AAC CTC AGC GAA CAG GAC  
 leu lys lys val lys lys gly asn ile thr trp ala ser phe asn leu ser glu gln asp  
 223/81  
 CTT GAC GAA TAC TAC AAC CAA TTC TCC AAT GCC GTT CTC TGG CCC GCT TTT CAT TAT CGG  
 leu asp glu tyr tyr asn gln phe ser asn ala val leu trp pro ala phe his tyr arg  
 283/101  
 CTC GAT CTG GTG CAA TTT CAG CGT CCT GCC TGG GAC GGC TAT CTA CGC GTA AAT GCG TTG  
 leu asp leu val gln phe gln arg pro ala trp asp gly tyr leu arg val asn ala leu  
 313/111

FIG. 11 A (Cont.)



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343/121 CTG GCA GAT AAA TTA CTG CCG CTG TTG CAA GAC GAT GAC ATT ATC TGG ATC CAC GAT TAT  
 leu ala asp lys leu leu pro leu leu gln asp asp ile ile trp ile his asp tyr  
 403/141 CAC CTG TTG CCA TTT GCG CAT GAA TTA CGC AAA CGG GGA GTG AAT AAT CGC ATT GGT TTC  
 his leu leu pro phe ala his glu leu arg lys arg gly val asn asn arg ile gly phe  
 463/161 TTT CTG CAT ATT CCT TTC CCG ACA CCG GAA ATC TTC AAC GCG CTG CCG ACA TAT GAC ACC  
 phe leu his ile pro phe pro thr pro glu ile phe asn ala leu pro thr tyr asp thr  
 523/181 TTG CTT GAA CAG CTT TGT GAT TAT GAT TTG CTG GGT TTC CAG ACA GAA AAC GAT CGT CTG  
 leu leu glu gln leu cys asp tyr asp leu leu gly phe gln thr glu asn asp arg leu  
 583/201 GCG TTC CTG GAT TGT CTT TCT AAC CTG ACC CCG CTC ACG ACA CGT AGC GCA AAA AGC CAT  
 ala phe leu asp cys leu ser asn leu thr arg val thr thr arg ser ala lys ser his  
 643/221 ACA GCC TGG GGC AAA GCA TTT CGA ACA GAA GTC TAC CCG ATC GGC ATT GAA CCG AAA GAA  
 thr ala trp gly lys ala phe arg thr glu val tyr pro ile gly ile glu pro lys glu  
 703/241 ATA GCC AAA CAG GCT GCC GCG CCA CTG CCG CCA AAA CTG CCG CAA CTT AAA GCG GAA CTG  
 ile ala lys gln ala ala gly pro leu pro pro lys leu ala gln leu lys ala glu leu

FIG. 11 B (Cont.)

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763/261 793/271  
 AAA AAC GTA CAA AAT ATC TTT TCT GTC GAA CCG CTG GAT TAT TCC AAA CGT TTG CCA GAG  
 lys asn val gln asn ile phe ser val glu arg leu asp tyr ser lys gly leu pro glu  
 823/281 853/291  
 CGT TTT CTC GCC TAT GAA GCG TTG CTG GAA AAA TAT CCG CAG CAT CAT GGT AAA ATT CGT  
 arg phe leu ala tyr glu ala leu leu glu lys tyr pro gln his his gly lys ile arg  
 883/301 913/311  
 TAT ACC CAG ATT GCA CCA ACG TCG CGT GGT GAT GTG CAA GCC TAT CAG GAT ATT CGT CAT  
 tyr thr gln ile ala pro thr ser arg gly asp val gln ala tyr gln asp ile arg his  
 943/321 973/331  
 CAG CTC GAA AAT GAA GCT GGA CGA ATT AAT GGT AAA TAC GGG CAA TTA GGC TGG ACG CCG  
 gln leu glu asn glu ala gly arg ile asn gly lys tyr gly gln leu gly trp thr pro  
 1003/341 1033/351  
 CTT TAT TAT TTG AAT CAG CAT TTT GAC CGT AAA TTA CTG ATG AAA ATA TTC CGC TAC TCT  
 leu tyr tyr leu asn gln his phe asp arg lys leu leu met lys ile phe arg tyr ser  
 1063/361 1093/371  
 GAC GTG GGC TTA GTG ACG CCA CTG CGT GAC GCG ATG AAC CTG GTA GCA AAA GAG TAT GTT  
 asp val gly leu val thr pro leu arg asp gly met asn leu val ala lys glu tyr val  
 1123/381 1153/391  
 GCT GCT CAG GAC CCA GCC AAT CCG GGC GTT CTT GTT CTT TCG CAA TTT GCG GGA GCG GCA  
 ala ala gln asp pro ala asn pro gly val leu val leu ser gln phe ala gly ala ala

FIG. 11 C (Cont.)

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1183/401  
 AAC GAG TTA ACG TCG GCG TTA ATT GTT AAC CCC TAC GAT CGT GAC GAA GTT GCA GCT GCG  
 asn glu leu thr ser ala leu ile val asn pro tyr asp arg asp glu val ala ala ala  
 1243/421  
 CTG GAT CGT GCA TTG ACT ATG TCG CTG GCG GAA CGT ATT TCC CGT CAT GCA GAA ATG CTG  
 leu asp arg ala leu thr met ser leu ala glu arg ile ser arg his ala glu met leu  
 1303/441  
 GAC GTT ATC GTG AAA AAC GAT ATT AAC CAC TGG CAG GAG TGC TTC ATT AGC GAC CTA AAG  
 asp val ile val lys asn asp ile asn his trp gln glu cys phe ile ser asp leu lys  
 1363/461  
 CAG ATA GTT CCG CGA AGC GCG GAA AGC CAG CAG CGC GAT AAA GTT GCT ACC TTT CCA AAG  
 gln ile val pro arg ser ala glu ser, gln gln arg asp lys val ala thr phe pro lys  
 1423/481  
 CTT GCG  
 leu ala

FIG. 11 D

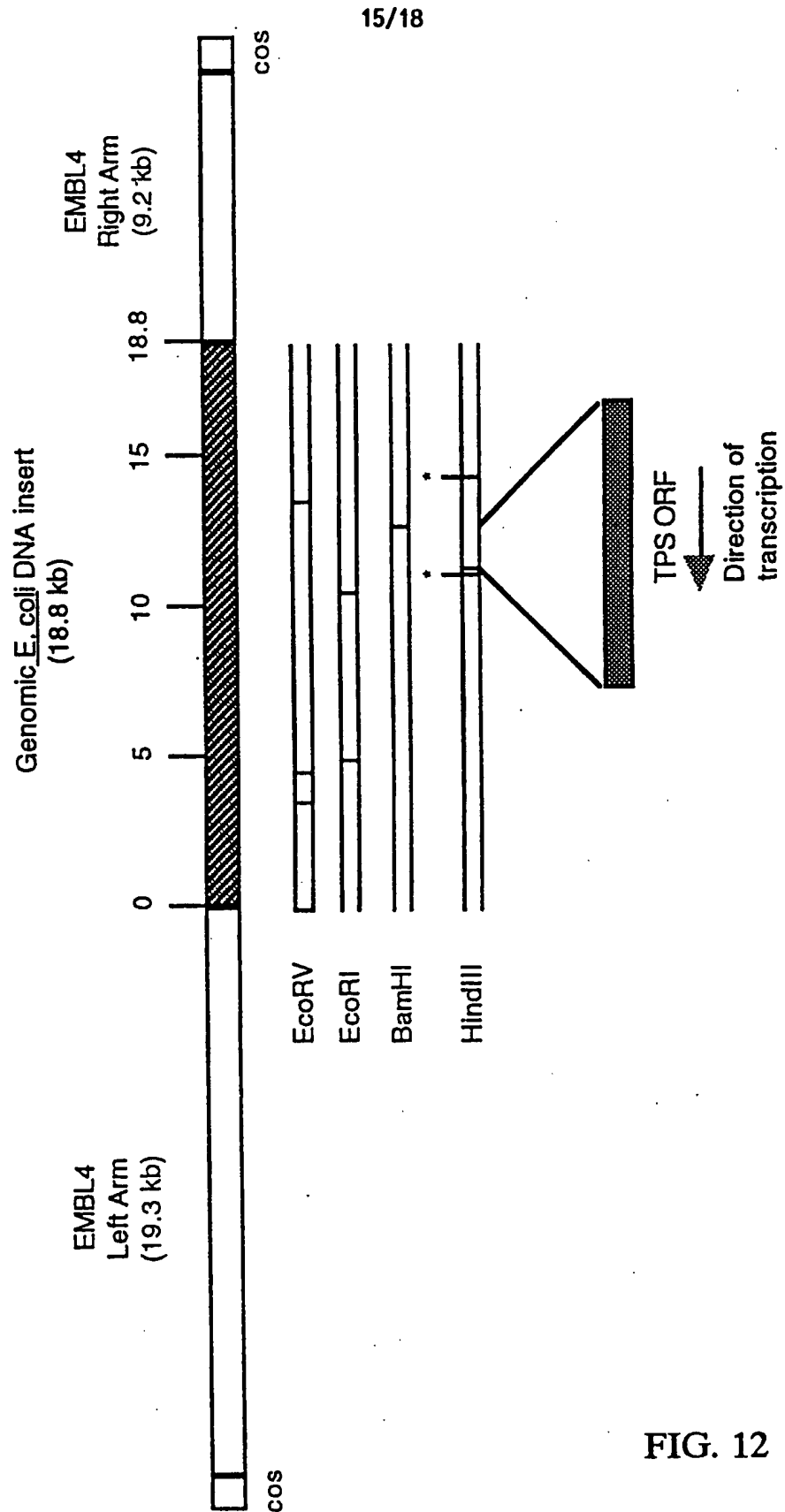


FIG. 12

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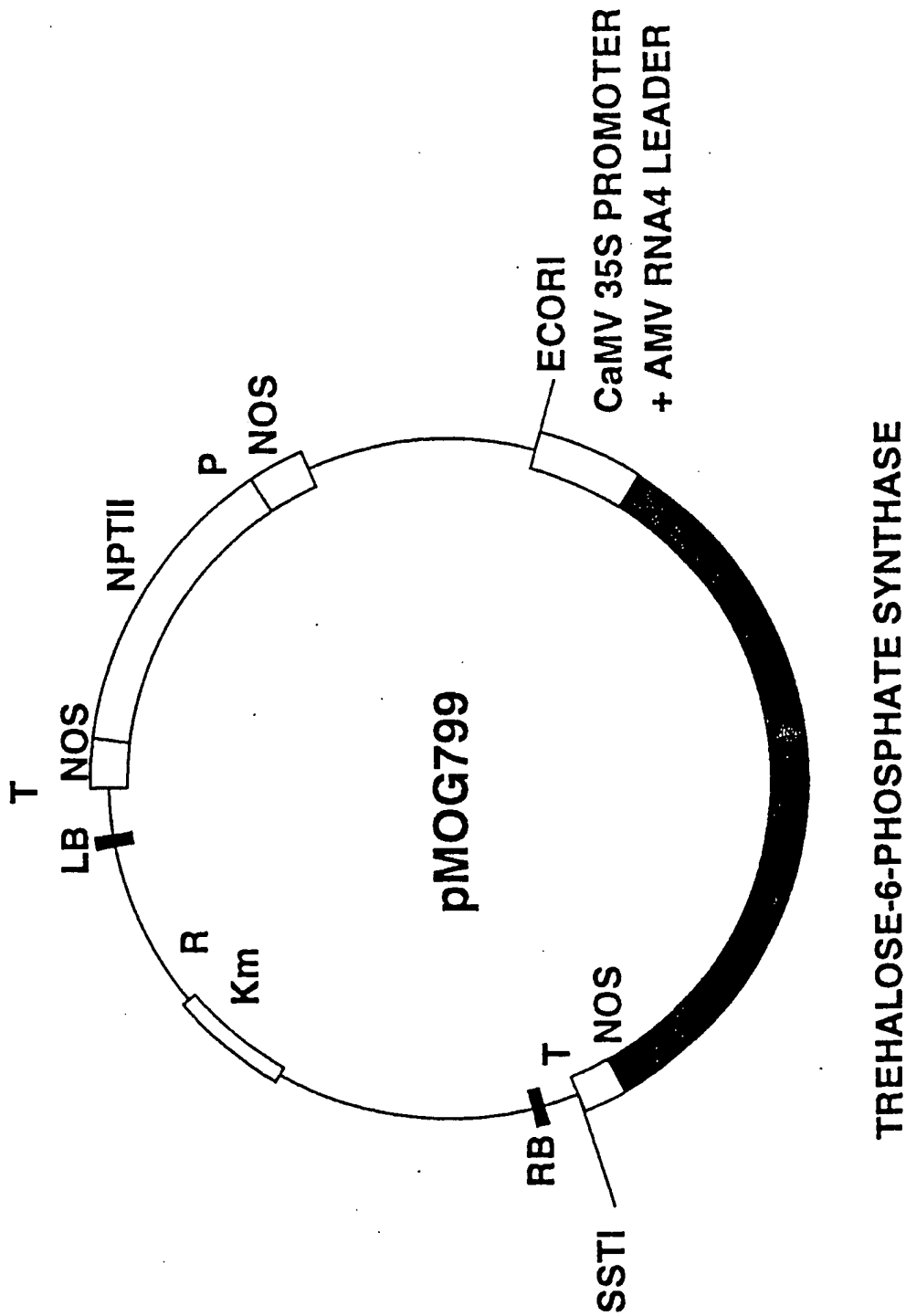


FIG. 13

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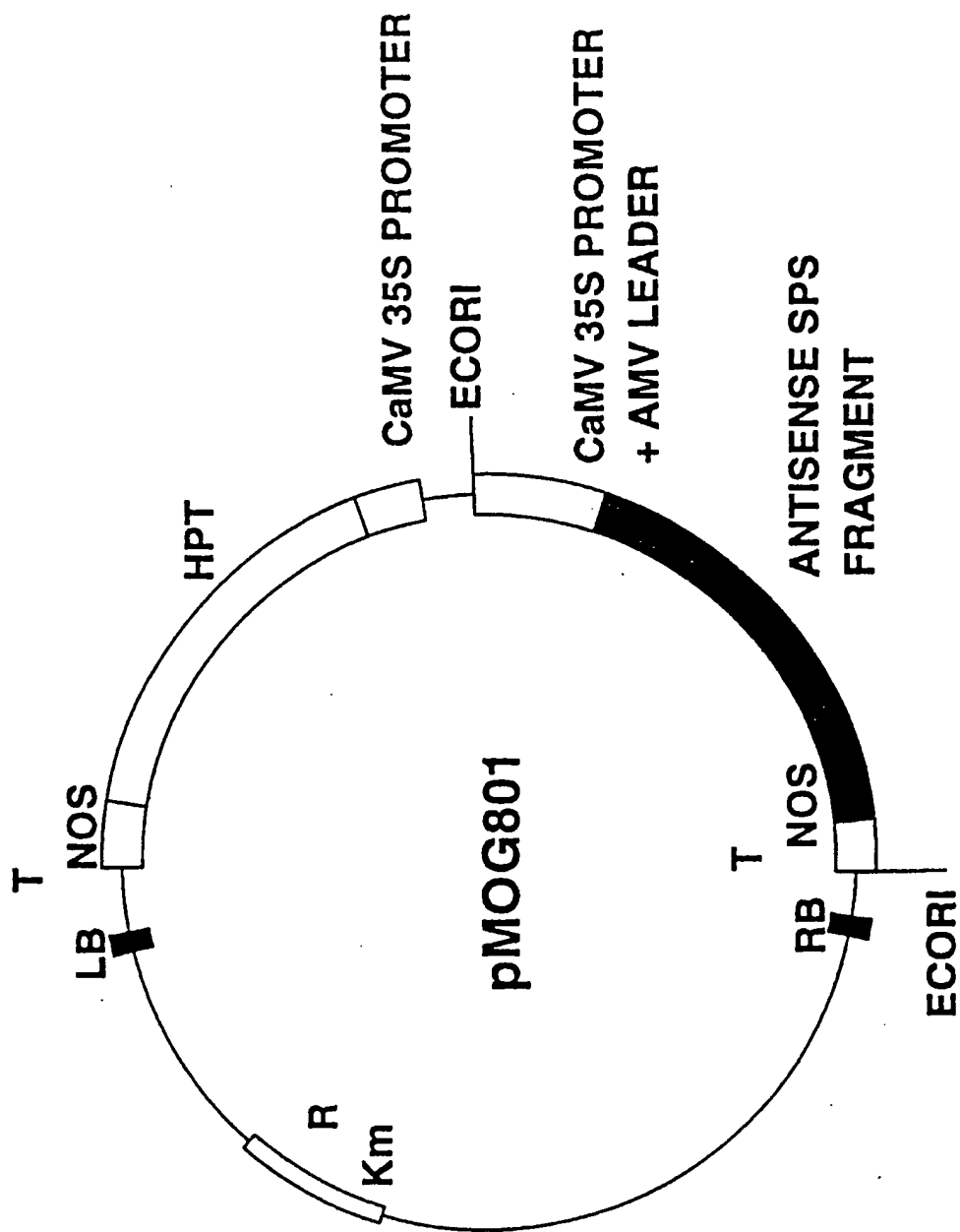


FIG. 14

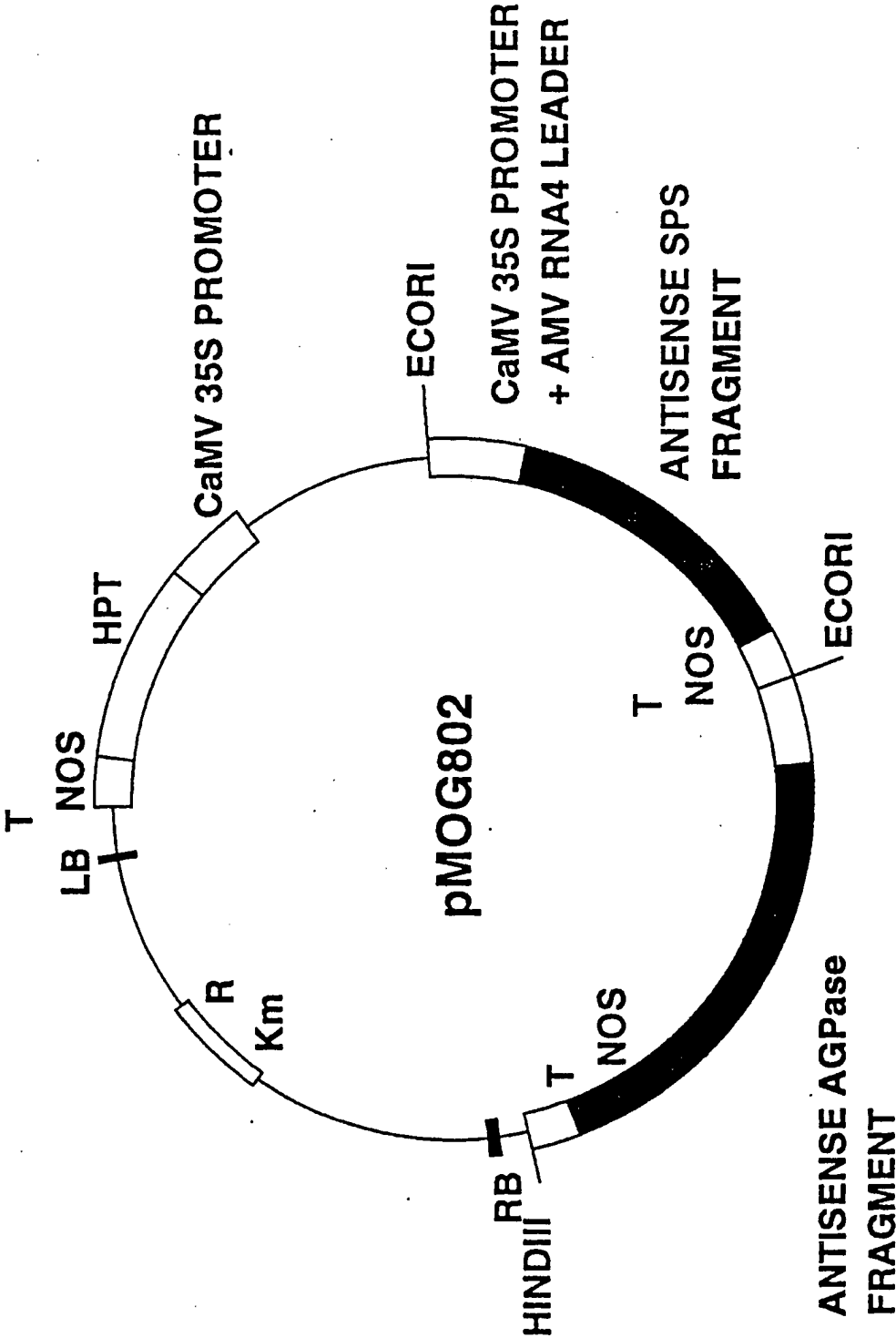


FIG. 15

## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/EP 93/02290

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/82 C12N15/54 C12N15/11 C12N1/21 A01H5/00  
C12N5/10 C12P19/12 A23L3/3562 A01N3/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A01H C12P A23L A01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>COMPTES RENDUES ACAD. SC. PARIS vol. 259, 20 July 1964 pages 635 - 637 QUILLET, M., ET AL. 'Sur l'accumulation concomitante du saccharose et du tréhalose chez plusieurs espèces de Sélaginelles indigènes et exotiques' see the whole document --- -/--</p>	34

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
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- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search

26 May 1994

Date of mailing of the international search report

24. 06. 94

Name and mailing address of the ISA

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Fax: (+31-70) 340-3016

Authorized officer

Maddox, A



## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/EP 93/02290

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EUROPEAN JOURNAL OF BIOCHEMISTRY vol. 209, no. 3 , November 1992 pages 951 - 959 BELL, W., ET AL. 'Characterization of the 56-kDa subunit of yeast trehalose-6-phosphate synthase and cloning of its gene reveal its identity with the product of CIF1, a regulator of carbon catabolite inactivation' see the whole document ---	34
X	YEAST vol. 8 , 1992 pages 183 - 192 GONZALES, M.I., ET AL. 'Molecular cloning of CIF1, a yeast gene necessary for growth on glucose' see the whole document ---	34
X	J. BACTERIOLOGY vol. 174, no. 3 , February 1992 pages 889 - 898 KAASEN, I., ET AL. 'Molecular cloning and physical mapping of the otsBA gene, which encode the osmoregulatory trehalose pathway of Escherichia coli: Evidence that transcription is activated by KatF (AppR)' cited in the application see the whole document ---	34-38
X	EMBL SEQUENCE DATABASE REL. ACC. NO. X69160 27 May 1993 ---	35-38
A	EP,A,0 451 896 (GIST-BROCADES) 16 October 1991 see the whole document ---	1-38
A	CURRENT BIOLOGY vol. 2, no. 11 , 1992 pages 594 - 596 TOMOS, D. 'Life without water' see page 596, left column, last paragraph ---	1-38
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA vol. 89 , April 1992 , WASHINGTON US pages 2600 - 2604 TARCZYNSKI, M.C., ET AL. 'Expression of a bacterial mtID gene in transgenic tobacco leads to production and accumulation of mannitol' see the whole document --- -/--	1-38

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 93/02290

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>PLANT PHYSIOLOGY. vol. 99, no. 1 , May 1992 , ROCKVILLE, MD, USA. page 27 TARCZYNSKI, M.C., ET AL. 'Evaluation of the concept of osmoprotection : Effect of mannitol production in transgenic tobacco' see abstract 162 ---</p>	1-38
A	<p>J. BIOTECHNOLOGY vol. 7, no. 1 , 1988 pages 23 - 32 COUTINHO, C., ET AL. 'Trehalose as cryoprotectant for preservation of yeast strains' see the whole document ---</p>	31,32
E	<p>WO,A,93 17093 (OY ALKO) 2 September 1993 see the whole document ---</p>	34
E	<p>EP,A,0 577 915 (ALGIST-BRUGGEMAN) 12 January 1994 see page 7, line 50 - line 54 -----</p>	34

**INTERNATIONAL SEARCH REPORT**

information on patent family members

Int: onal Application No

**PCT/EP 93/02290**

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0451896	16-10-91	AU-B- 636022	08-04-93
		AU-A- 7378291	03-10-91
		JP-A- 5184353	27-07-93
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WO-A-9317093	02-09-93	AU-B- 3500993	13-09-93
-----			
EP-A-0577915	12-01-94	NONE	
-----			